Nicotinamide Nucleotides in the Erythrocytes of Patients Suffering from Pellagra

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(Received 29 December 1964)

1. The nicotinamide nucleotide concentrations in the erythrocytes of subjects suffering from pellagra (pellagrins) were not lower than those in normal subjects, but the ability of erythrocytes to synthesize these nucleotides *in vitro* was significantly lower in pellagrins. 2. The oral administration of 10 g. of L-leucine daily for 5 days depressed the nicotinamide nucleotide-synthesizing ability of erythrocytes both in normal subjects and in pellagrins. This was not accompanied by changes in the nucleotide concentration in erythrocytes. 3. Quinolinic acid brought about a significant inhibition of the synthesis of nicotinamide nucleotides *in vitro*. Such inhibition was partially overcome by increasing the concentration of nicotinic acid in the medium.

Pellagra has been traditionally associated with maize diets, but it was reported by Gopalan & Srikantia (1960) that it also occurs in an endemic form in a population whose staple food is jowar (*Sorghum vulgare*). A feature common to both maize and jowar is the relatively high amount of leucine. It has been shown by Bhavani Belavady, Srikantia & Gopalan (1963) that supplements of pure L-leucine to diets marginal in protein bring about significant alterations in nicotinic acid-tryptophan metabolism. These observations suggested a possible role of amino acid imbalance in the pathogenesis of pellagra.

A possible effect of amino acid imbalance may be an interference with the utilization of nicotinic acid for the formation of nucleotides, the functional form of nicotinic acid. In the present study, nucleotide concentration and its synthesis *in vitro* have been studied in patients suffering from pellagra (pellagrins) and in normal subjects. The effects of leucine supplements on the concentration of nucleotide in erythrocytes and the synthesis of nucleotides by erythrocytes *in vitro* have also been investigated. Previous studies in these Laboratories had shown that one of the striking features of leucine supplementation was an increase in the excretion of quinolinic acid in urine (Bhavani Belavady *et al.* 1963). Since it became apparent that leucine supplements inhibited the synthesis of nucleotides by erythrocytes *in vitro*, the possible relationship between nucleotide synthesis *in vitro* and quinolinic acid was also investigated.

MATERIALS AND METHODS

Subjects and diets. Forty-seven normal subjects and 25 pellagrins aged 25–40 years were investigated. Samples of venous blood were taken from subjects kept without food for 12 hr. for the determination of nucleotide concentrations in the erythrocytes. Eight pellagrins and eight normal subjects were placed on a standard diet based on rice, wheat flour, legumes and vegetables, which provided approx. 2400 kcal., 50 g. of protein and 50 g. of fat. The protein was derived entirely from vegetable sources and contributed about 8% of the total calories. Each subject received a daily supplement of 10 g. of L-leucine with the main meal of the day for five days. Samples of blood were drawn on the sixth day. Five days later, another sample of blood was drawn for analysis.

Analytical procedures. Nicotinamide nucleotide concentrations were determined by the method of Levitas, Robinson, Rosen, Huff & Perlzweig (1947). The synthesis of nicotinamide nucleotides by erythrocytes *in vitro* was measured by a method based on that of Leder & Handler (1951) and Preiss & Handler (1957). To determine the effect of quinolinic acid on nucleotide synthesis, erythrocytes were obtained from three normal subjects and 0, 0.5, 1.0, 2.0, 2.5 and 5.0 μ moles of quinolinic acid added to the medium, which contained either 0.5 or 1.0 μ mole of nicotinic acid. The synthesis of nucleotides was measured as described above.

RESULTS

Nucleotide concentrations and synthesis in erythrocytes. The mean concentrations of nucleotides in erythrocytes were similar in the normal subjects and pellagrins. The rate of synthesis, however,

was significantly lower in pellagrins ($P < 0.05$) (Table 1).

Effect of leucine on nucleotide concentrations and synthesis. Supplementation with leucine had no effect on the nucleotide concentration in erythrocytes either in normal subjects or in pellagrins. The total synthesis of nicotinamide nucleotides in erythrocytes, however, was significantly lowered in both groups to about 60% of the initial values ($P < 0.01$) after leucine supplementation. Withdrawal of leucine restored the synthesis to the pre-leucine amounts (Table 2).

Effect of quinolinic acid on nucleotide synthesis. The presence of quinolinic acid in the medium inhibited nucleotide synthesis, the inhibition being progressively more marked from 0.5 to 2.0 μ moles. Maximal inhibition occurred at 2.0 μ moles, when the content of nicotinic acid was 0.5 or 1.0 μ mole, but with 1.0 μ mole of nicotinic acid the inhibition

at all concentrations of quinolinic acid was less than that with 0.5 μ mole (Fig. 1).

DISCUSSION

The observation that the nicotinamide nucleotide contents of erythrocytes of pellagrins were not different from those observed in normal subjects indicates that nucleotide concentration of erythrocytes is a poor index of nicotinic acid status. These results are in keeping with the observations made by Axelrod, Spies & Elvehjem (1941) that the NAD concentrations in erythrocytes in mild or moderately severe cases of pellagra are not lowered. This is somewhat similar to the finding that, even in severe cases of pellagra, blood nicotinic acid concentrations do not fall (Klein, Perlzweig & Handler, 1942; Salamon, Jetic & Karamchmedovic, 1960; Gopalan & Srikantia, 1960). However, the ability of erythrocytes to synthesize nucleotides *in vitro* was significantly lower in pellagrins. The presence of normal concentration of nucleotides in the face of lowered capacity to synthesize needs explanation.

Table 1. Concentrations of nicotinamide nucleotides and synthetic activity *in vitro* in erythrocytes of normal subjects and pellagrins

Erythrocytes were separated, washed four times with 4 vol. of Ringer phosphate buffer, pH 7.4, and made up to twice the volume by adding an equal volume of buffer. To 1.5 ml. of suspension were added 10 μ moles of glutamine, 125 μ moles of glucose, 0.5 μ mole of nicotinic acid, 20 units of crystalline penicillin and 40 units of streptomycin. The mixture was incubated at 37° for 24 hr. Trichloroacetic acid (10%, w/v) (1.9 ml.) was added at the end of the incubation and total nucleotides were determined in the supernatant. The entire procedure was carried out under sterile conditions. The incubation medium was prepared at 4°. Values are given as means $\pm$ s.e.m., with the numbers of subjects in parentheses.

	Nicotinamide nucleotides (mg./100 ml. of erythrocytes)	
	Actual concn.	Synthetic activity
Normal subjects	4.75 $\pm$ 0.14 (47)	11.69 $\pm$ 0.93 (12)
Pellagrins	4.91 $\pm$ 0.23 (25)	7.84 $\pm$ 1.31 (12)

Fig. 1. Effect of added quinolinic acid on the synthesis of nicotinamide nucleotides by human erythrocytes at different concentrations of nicotinic acid as substrate: O, 1 μ mole of nicotinic acid; Δ , 0.5 μ mole of nicotinic acid.

Table 2. Effect of leucine on erythrocyte nicotinamide nucleotides in normal subjects and pellagrins

Values are given as means $\pm$ s.e.m., with the numbers of subjects in parentheses.

	Nicotinamide nucleotides (mg./100 ml. of erythrocytes)					
	Initial		5 days after leucine		5 days after leucine withdrawal	
	Actual concn.	Synthetic activity	Actual concn.	Synthetic activity	Actual concn.	Synthetic activity
Normal subjects	4.7 $\pm$ 0.42 (8)	11.8 $\pm$ 0.90 (5)	4.3 $\pm$ 0.29 (8)	6.9 $\pm$ 1.10 (5)	4.5 $\pm$ 0.41 (8)	11.2 $\pm$ 0.07 (5)
Pellagrins	4.9 $\pm$ 0.38 (8)	7.6 $\pm$ 1.80 (5)	4.3 $\pm$ 0.56 (8)	4.7 $\pm$ 1.66 (5)	5.3 $\pm$ 0.44 (8)	8.1 $\pm$ 1.49 (5)

Synthesis *in vitro* represents the capacity of mature erythrocytes to synthesize nucleotides, whereas the concentration *in vivo* may represent formation during development of the erythrocytes from precursor cells. The finding of normal concentrations *in vivo* may therefore indicate that the synthesizing capacity of pre-erythrocytes, unlike that of mature erythrocytes, in pellagrins is unaltered. Further, since the conditions of the study probably indicate the maximal capacity of the erythrocytes to synthesize nucleotides from precursor compounds, it seems that the normal concentrations of nucleotides in erythrocytes, which are lower than the maximal, can be maintained in spite of some decrease in the synthesizing capacity; also, the concentration of a metabolite in a tissue depends not only on the rate of synthesis but also on the rate of utilization or breakdown.

The method employed above for the determination of nicotinamide nucleotides measures NAD, NADP, *N'*-methylnicotinamide and NMN. All the nucleotide synthesized *in vitro* is NAD (Preiss & Handler, 1957), but there may be differences in the type of nucleotides formed by the erythrocytes of normal subjects and of pellagrins.

The observation that leucine significantly lowered the nucleotide-synthesizing capacity of erythrocytes both in normal subjects and in pellagrins supports suggestions (Bhavani Belavady *et al.* 1963) that nicotinic acid-tryptophan metabolism and leucine are interrelated. One of the striking changes induced

by the administration of leucine is the increase in the urinary excretion of quinolinic acid (Bhavani Belavady *et al.* 1963). This increase suggests higher amounts of quinolinic acid in circulation, probably as a result of greater amounts formed in the liver, a major site of conversion of tryptophan into quinolinic acid. Experiments on the synthesis of nucleotides *in vitro* with quinolinic acid in the medium were done to determine whether the lowered nucleotide synthesis that leucine brought about was mediated through increased amounts of quinolinic acid. The results of these studies support such a possibility. The mechanism by which the inhibitory effect of quinolinic acid occurs, however, needs elucidation.

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