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Pattern of Nicotinamide Nucleotides in the Erythrocytes of Pellagrins[1]

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IT WAS REPORTED earlier from these Laboratories that the concentration of nicotinamide nucleotides in the erythrocytes of subjects suffering from pellagra was not different from that in normal subjects, but the ability of erythrocytes to synthesize the nucleotides in vitro was significantly lower in pellagrins (1). It was also observed that oral administration of leucine considerably depressed the ability of erythrocytes to synthesize in vitro nicotinamide nucleotides both in normal subjects and in pellagrins but did not bring about any alterations in the total nucleotide concentration. These apparently paradoxical results suggested that the type of nucleotides in the erythrocytes of pellagrins may be different from that in normal subjects. Fractionation of the nucleotides was, therefore, carried out to examine this possibility. In addition, the nature of nucleotides synthesized in vitro in the erythrocytes of normals and pellagrins was also examined.

MATERIALS AND METHODS

Fasting samples of venous blood were obtained from 12 normal subjects and 20 pellagrins for the determination of total nicotinamide nucleotides and fractions. In 10 of these pellagrins blood samples were again obtained after intramuscular administration of 300 mg nicotinic acid daily for 5 days. During this period they received a diet that provided daily about 2,400 kcal, 50 g of protein, and 50 g of fat.

Erythrocytes were separated, washed, and made up to known volume with 0.1 M Ringer phosphate buffer, pH 7.4. To 1.5 ml of this cell suspension, 0.5 ml of 12% perchloric acid was added to precipitate proteins. After centrifugation, the supernatant was adjusted to pH 7.0 with 2 N KOH and filtered. One-tenth milliliter of filtrate was spotted on Whatman no. 1 filter paper and irrigated overnight in a solvent system of seven parts of 95% ethyl alcohol and three parts of 1 M ammonium acetate (pH 5.0) by the ascending technique (2). The nucleotides were located as fluorescent spots (3). The spots of nicotinamide mononucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) were identified using authentic samples. A third spot which was located above the NAD and NADP spots and appeared only in the erythrocytes of pellagrins was identified as nicotinamide mononucleotide (NMN) by its ultraviolet absorption spectra (maxima, 264 mμ) and by paper chromatography in a 2:1 pyridine–water system. This was confirmed by two-dimensional paper chromatography using ammonium acetate and alcohol (R_F, 0.25) in the first run and pyridine water in the second run (R_F, 0.4). Further, the R_F value of the spot was identical with that of authentic samples of NMN.

The spots were eluted with 1 ml of 0.01 N HCl and estimated fluorometrically (4) using pure samples as the standard. In the chromatograms of normal subjects, the area of paper corresponding to the NMN spot was eluted to determine whether NMN was present.

It was found that 95% of the spotted quan-

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TABLE I

Fractionation of nicotinamide nucleotides in the erythrocytes of normals and pellagrins

	Number of Subjects	Erythrocytes, mg/100 ml				% NMN	% NAD	% NADP	NAD/NADP
		Total nicotinamide nucleotides	NMN	NAD	NADP				
Normals	12	5.02 ± 0.44	[a]	2.48 ± 0.27	1.43 ± 0.21		65.42 ± 2.13	34.58 ± 2.13	1.99 ± 0.23
Pellagrins	20	4.97 ± 0.66	0.73 ± 0.09	2.13 ± 0.19	1.00 ± 0.17	18.96 ± 2.11	53.40 ± 2.53	27.63 ± 2.45	2.15 ± 0.22
Pellagrins after treatment	10	4.97 ± 0.66	0.18 ± 0.04	3.01 ± 0.45	1.37 ± 0.14	6.50 ± 2.04	62.80 ± 2.79	30.70 ± 2.45	2.18 ± 0.21

Values are means ± SE. [a] Not detectable except in three subjects.

tity was eluted out by this method and that as low as 1 μg of the nucleotide could be estimated. The recovery of the added authentic sample to the filtrate was 90%.

RESULTS

The amounts of the various nucleotides present in the erythrocytes of normal subjects and of pellagrins are given in Table I. The erythrocytes of pellagrins had considerable amounts of NMN, which were not present in the erythrocytes of most normal subjects. It was, however, present to the extent of 2–5% of total nucleotides in three subjects. Levels of NAD and NADP in the erythrocytes of pellagrins were lower than in normal subjects, but these differences were not statistically significant. The ratio of NAD to NADP was similar in both the groups. Administration of nicotinic acid to pellagrins brought about a significant reduction in NMN concentration and an increase in both the NAD and NADP levels. These increases, however, were not statistically significant. Total nicotinamide nucleotide concentration remained unchanged after treatment. Fractionation of nucleotides formed during in vitro incubation of the erythrocytes (1) indicated that almost all of the newly synthesized nucleotides were NAD, both in the normal and in the pellagrin (Table II).

TABLE II

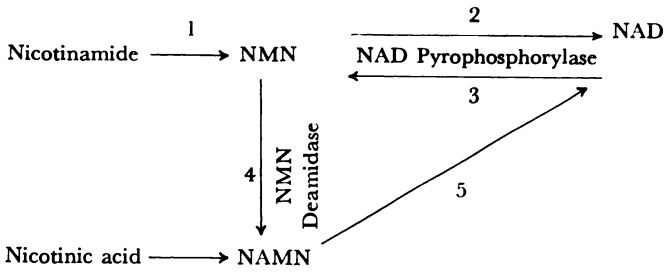
Separation of nicotinamide nucleotides of erythrocytes before and after in vitro synthesis

	Erythrocytes, mg/100 ml[a]		
	NMN	NAD	NADP
Normal subjects			
Before synthesis		2.5	1.4
After synthesis	0.10	10.4	1.5
Pellagrins			
Before synthesis	0.8	1.8	0.9
After synthesis	1.0	5.4	1.1

[a] Average of three determinations. Nucleotides were separated by paper chromatograph. Details given in text. Conditions of incubation have been described earlier (Raghuramulu et al. (1)).

DISCUSSION

The nucleotides separated on paper accounted for 75% of the estimated total nucleotides in the normal subjects as well as in the pellagrins. The alkali-acetone condensation method (3) employed here estimated all the *N*-substituted nicotinamide compounds. However, the spots identified and eluted from the paper chromatograms may not account for all the nucleotides because nucleotides other than NAD and NADP present in amounts insufficient to be visualized as spots would not be estimated by this procedure. The



SCHEME 1. Some of the reactions involved in the synthesis and breakdown of NAD.

values of NAD and NADP obtained in normal subjects in the present investigation are in close agreement to those reported by other workers using different methods (5–7).

The significant observation made here is that though the total nucleotide content of erythrocytes of pellagrins is similar to that of normal subjects, the proportion of the different nucleotides is considerably different. Erythrocytes of pellagrins were found to contain significantly higher amounts of NMN and lower amounts of NAD and NADP, as compared to the erythrocytes of normal subjects.

Increased amounts of NMN in the erythrocytes of pellagrins may be due to one of several reasons. Some of the reactions that are involved in the synthesis of NAD are indicated in Scheme 1. Nicotinamide mononucleotide may accumulate because of *a*) an accelerated rate of conversion of nicotinamide to NMN (*reaction 1*), *b*) a slower rate of conversion of NMN to either NAD (*reaction 2*) or to NAMN (*reaction 4*), or *c*) an accelerated rate of breakdown of NAD to NMN (*reaction 3*). It has been demonstrated that nicotinamide can serve as a precursor for the in vitro synthesis of NMN by human erythrocytes (8), but the K_m value for nicotinamide is very high, suggesting thereby that NMN is not a physiological intermediate in the biosynthesis of NAD (2). Similarly, the equilibrium of the reaction between NMN and and NAD is such that it contributes more to the breakdown of NAD than to its synthesis (9). The NMN formed as a result of NAD breakdown, however, can be reutilized for NAD synthesis by conversion into nicotinic acid mononucleotide (NAMN) by the action of NMN deamidase (10). Nicotinamide mononucleotide deamidase has been shown to be present in mammalian liver, but its presence in human erythrocytes has still to be established.

The observation made here that levels of NAD are lower in the erythrocytes of pellagrins than in normals strongly suggests that the increased amounts of NMN are due to enhanced breakdown of NAD. The demonstrated lowered capacity of erythrocytes of pellagrins to synthesize NAD (1) and the accumulation of NMN in these erythrocytes may indeed be related. Whether lowered activity of NMN deamidase is also involved, thus affecting the reutilization of NMN for NAD synthesis, has to await the demonstration of the presence of this enzyme in human erythrocytes. These aspects are now currently being studied.

SUMMARY

Nicotinamide nucleotides in the erythrocytes of normal human subjects and of patients suffering from pellagra were separated by paper chromatography and their concentrations determined. Though no differences were observed in the concentration of total nicotinamide nucleotides in erythrocytes of pellagrins and normals, significant differences were observed with regard to concentration of the individual

nucleotides. The erythrocytes of pellagrins had significantly higher amounts of NMN than the erythrocytes of normal subjects, while levels of NAD and NADP tended to be lower in the pellagrins as compared to normals.

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