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THE INCORPORATION OF NICOTINIC ACID AND OF NICOTINAMIDE INTO THE PYRIDINE NUCLEOTIDES OF ERYTHROCYTES AND RETICULOCYTES OF RABBITS *IN VITRO* *

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There is abundant evidence that loss of intracellular structure and alteration in metabolic activity occur as mammalian reticulocytes mature to become nonreticulated erythrocytes (2). Loss of the ability to synthesize heme (3), proteins (3, 4), lipids (5–7), purine nucleotides (8), and pyrimidine nucleotides (9) from small molecule precursors accompanies the disappearance of mitochondria, ribosomes, and ribonucleic acid from reticulocytes. An intact Krebs tricarboxylic acid cycle and a complete cytochrome system are not present in the mature mammalian erythrocyte (10). The concentration of ATP is approximately 2.5 times greater in rabbit reticulocytes than in the mature erythrocytes of rabbits (11). Further changes, including a progressive decrease in the activity of several enzymes of the glycolytic pathway (12) and of the hexose monophosphate shunt pathway (12, 13) and in the concentration of ATP (12, 14), are associated with aging of mature mammalian erythrocytes *in vivo*. The concentrations of DPN and of TPN of avian and human erythrocytes were reported to decrease during storage *in vitro* (15), and the concentration of DPN in human erythrocytes was found to decrease with aging *in vivo* (16). It was of interest, therefore, to study in more detail the metabolism of the pyridine nucleotides in mammalian erythrocytes as a function of the age of the cells. The present report provides evidence for a difference in the ability of reticulocytes and of mature erythrocytes of rabbits to incorporate radioactive nicotinic acid and nicotinamide into DPN and TPN *in vitro*.

MATERIALS AND METHODS

Whole blood, anticoagulated with heparin, was obtained from normal, adult, white New Zealand rabbits. In order to obtain blood enriched with reticulocytes, rabbits were made severely anemic by the administration of acetylphenylhydrazine, 10 mg per kg daily for 5 days. These rabbits were exsanguinated 2 days after the final injection. The blood was centrifuged immediately, the plasma and buffy coat were removed, and the cells were washed three times with 4 to 5 vol of cold 0.9% sodium chloride solution. Flasks for incubation were prepared to contain 10 ml of erythrocytes or reticulocytes, 1.6 to 1.7 μmoles and 9.4 to 10.0 μc of radioactive nicotinic acid or nicotinamide, 870 μmoles of glucose, 140 μmoles of glutamine, 330 μmoles of inorganic phosphate, 9 mg of penicillin, and 9 mg of streptomycin in a total volume of 15 ml. The specific activity of the nicotinic acid-7-C^{14} was 5.9 μc per μmole and that of the nicotinamide-7-C^{14} was 5.6 μc per μmole.[1] In experiments in which different concentrations of nicotinic acid were employed, the specific activity was reduced proportionally by the addition of unlabeled compound. The incubation medium was modeled after the one found to result in optimal synthesis of DPN from nicotinic acid by human erythrocytes *in vitro* (17). The pH of the cell suspensions was 7.3 before incubation and 7.1 to 7.2 after 4 to 8 hours of incubation. Cultures of the cell suspensions after incubation demonstrated the absence of bacterial contamination. The actual volume of packed washed cells was determined with hematocrits. Erythrocyte, leu-

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[1] Radiocarbon-labeled compounds were obtained from New England Nuclear Corp., Boston, Mass.

kocyte, and reticulocyte counts were performed in duplicate by routine hematological methods.

The flasks were incubated for 4 hours, unless otherwise indicated, at 37° C in air in a Dubnoff metabolic shaker-incubator. After incubation, the cells were again washed three times with cold isotonic sodium chloride solution. Nucleotides were extracted successively with 2 vol of cold 10% trichloroacetic acid and 2 vol of cold 5% trichloroacetic acid. The filtered, pooled extracts were added to 5 vol of acetone and were kept at −20° C for 16 to 24 hours (18). The precipitate was collected by centrifugation at 4° C, washed once with cold acetone and twice with cold ether, and was dried in a stream of nitrogen.

The dried precipitate was redissolved in 10 ml of water, and insoluble material was removed by centrifugation. The clear supernatant solution was loaded on a column of washed Dowex 1-X2 resin (formate cycle, 200 to 400 mesh, 10.0 cm × 1.8 cm^2). The nucleotides were removed from the resin by extended gradient elution with increasing concentrations of formic acid, as described by Hurlbert, Schmitz, Brumm, and Potter (19) and as applied by Mills and Summers (20). Appropriate fractions were pooled, and formic acid was removed by evaporating the solutions to dryness under reduced pressure. The dried material was redissolved in a small volume of water. Samples of these solutions were applied to Whatman no. 1 paper, and the chromatograms were developed in an ascending system in two different solvents for 16 to 20 hours: *1*) system no. I (21) and *2*) system C (22). Nucleotides were eluted from the papers with 0.01 N hydrochloric acid. The concentrations of DPN or TPN in the eluates were determined from the absorbancy at 260 mμ, after correction for an appropriate paper blank, by utilizing a millimolar extinction coefficient (E_{mM}) of 18.5 (21). These values were in good agreement with values obtained by fluorometric analysis (23). Samples of the eluates were plated as infinitely thin films, and the radioactivity was determined in a gas-flow counter. All samples were counted for a sufficient length of time to assure a standard error no greater than 5%.

The adequacy of the isolation procedure of DPN and TPN was based upon several criteria. The positions in the column chromatographic profile of nicotinic acid, nicotinamide, nicotinamide mononucleotide, DPN, AMP (adenosine monophosphate), TPN, and ADP were determined. The pyridine nucleotides were further identified by the development of a fluorescent methyl-ethyl ketone addition product (23), and by specific enzymatic assays, with alcohol dehydrogenase for DPN and with isocitric dehydrogenase for TPN (23, 24). The R_f values, determined from the paper chromatograms, and the ratios of absorbancy at several wave lengths were in good agreement with values determined for DPN and TPN standards.

In order to identify the portion of the pyridine nucleotide molecule that contained the radioactive label, appropriate fractions were heated in a boiling water bath for 20 minutes with equal volumes of concentrated ammonium hydroxide. The products resulting from alkaline hydrolysis (22) were identified by paper chromatography. Eighty-six to 94% of the radioactivity was located in the area corresponding to nicotinamide. The remaining radioactivity was present in the area corresponding to nicotinic acid. When nicotinamide-7-C^{14} was heated with alkali and then analyzed by paper chromatography, an identical distribution of radioactivity was observed.

The concentrations of total oxidized pyridine nucleotides in extracts of erythrocytes or reticulocytes prepared before and after incubation were determined fluorometrically (23). One-ml samples of the cell suspensions were added to 2 ml of cold 7% trichloroacetic acid, and filtrates were used for the assays.

RESULTS

Incorporation of nicotinic acid. The results of a typical experiment with mature erythrocytes of rabbits are shown in Figure 1 A, where a column chromatographic profile is depicted, together with the specific activities of the isolated DPN and TPN. Significant incorporation of nicotinic acid into DPN occurred after 4 hours of incubation, but there was no radioactivity in the TPN. The average specific activity of the DPN isolated in seven experiments was 4,100 cpm per μmole (range: 3,030 to 4,900 cpm per μmole). An increase in the specific activity of the DPN as a function of the length of time of incubation was observed between 0 and 8 hours (Figure 2). After 8 hours of incubation, a small but significant incorporation of nicotinic acid into TPN was noted in two separate experiments. The specific radioactivity of the DPN was consistently 18 to 20 times greater than that of the TPN after the longer period of incubation.

The incorporation of nicotinic acid-7-C^{14} into the pyridine nucleotides of reticulocytes obtained from acetylphenylhydrazine-treated rabbits differed markedly from that observed with mature erythrocytes (Figure 1 B). After 4 hours of incubation, the specific activity of the DPN isolated from these immature cells was almost four times greater than that of the DPN obtained from mature erythrocytes. Although significant labeling of TPN had occurred, the specific activity of the TPN was only about one-tenth that of the DPN. In six separate experiments, the specific activity of the DPN averaged 19,400 cpm per

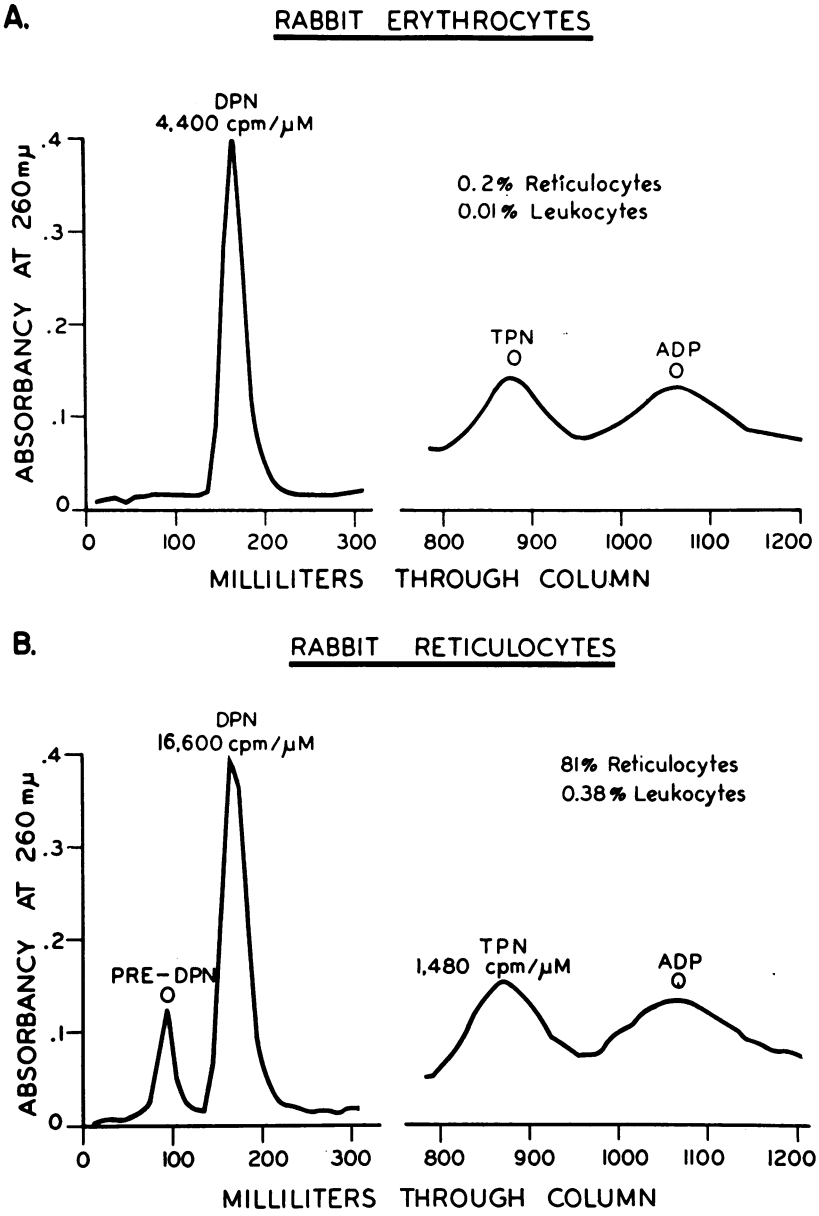

FIG. 1. COLUMN CHROMATOGRAPHIC SEPARATION OF NUCLEOTIDES FROM A) MATURE ERYTHROCYTES AND B) RETICULOCYTES OF RABBITS. The cells were incubated for 4 hours with nicotinic acid-7-C^{14} and substrates as described in the text. The specific activities of the DPN and TPN isolated after paper chromatography of the pooled column fractions are indicated above the peaks. The ADP and pre-DPN fractions were found to be free of radioactivity after paper chromatography.

μmole (range: 14,300 to 33,400 cpm per μmole) and that of the TPN averaged 1,500 cpm per μmole (range: 1,000 to 2,500 cpm per μmole). The results of paper chromatography and spectral analyses suggest that the fractions in the chromatographic profile labeled pre-DPN (Figure 1 B) contained cytidine and deoxycytidine diphosphates. These compounds have been noted by Mills (25) to be present in mature erythrocytes of man. The meaning of this ultraviolet light-absorbing peak which was present in the chromatographic profile obtained with rabbit re-

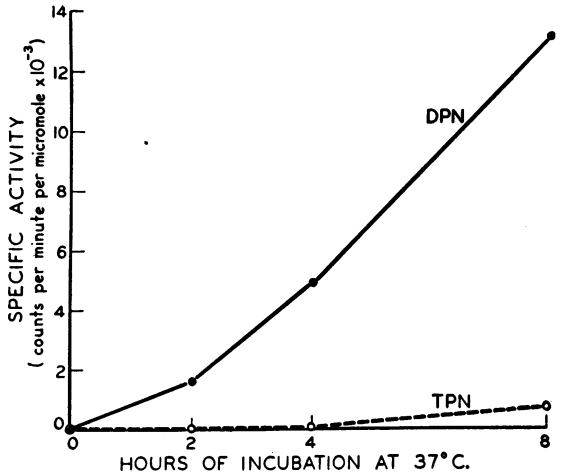

FIG. 2. INCORPORATION OF NICOTINIC ACID-7-C^{14} INTO THE PYRIDINE NUCLEOTIDES OF MATURE ERYTHROCYTES OF RABBITS AFTER INCUBATION AT 37° C.

ticulocytes, but not with mature erythrocytes, is obscure.

It was not possible to remove leukocytes completely from erythrocytes and, especially, from reticulocytes by washing with isotonic sodium chloride solution. It was deemed necessary, therefore, to investigate the contribution of leukocytes to the labeled pyridine nucleotides. Purposeful contamination of mature erythrocytes with a large number of leukocytes and with a slight increase in reticulocytes did not alter the specific activity of the isolated DPN or TPN (Table I). Similarly, doubling the number of white cells in an experiment with reticulocytes did not increase the specific activity of the DPN and TPN.

TABLE I

*The effect of added leukocytes on the incorporation of nicotinic acid-7-C^{14} into pyridine nucleotides of erythrocytes and reticulocytes of rabbits**

	Leukocytes	Reticulocytes	Specific activity DPN	Specific activity TPN
	%	%	*cpm/μmole*	
Erythrocytes	0.01	0.2	4,425	0
Erythrocytes plus leukocytes	0.53	2.0	4,030	0
Reticulocytes	0.38	81	16,600	1,480
Reticulocytes plus leukocytes	0.83	82	17,400	1,600

* Incubation for 4 hours at 37° C.

In other experiments, mature erythrocytes and reticulocytes of rabbits were incubated for 4 hours with increasing concentrations of nicotinic acid (Table II). The relative specific activity (RSA) of the isolated DPN varied directly in both types of cells as the concentration of nicotinic acid was increased. However, the extent of incorporation into the DPN of reticulocytes, at comparable concentrations of nicotinic acid, was always about five times greater than the extent of incorporation into the DPN of mature erythrocytes. An in-

TABLE II

*Relative specific activities of DPN and TPN and concentration of total oxidized pyridine nucleotides after incubation of erythrocytes and reticulocytes of rabbits with several concentrations of nicotinic acid and one concentration of nicotinamide**

Nicotinic acid	Nicotinamide	Specific activity of precursor	Erythrocytes† Relative specific activity‡ DPN	Erythrocytes† Relative specific activity‡ TPN	Erythrocytes† Total oxidized PN§	Reticulocytes† Relative specific activity‡ DPN	Reticulocytes† Relative specific activity‡ TPN	Reticulocytes† Total oxidized PN§
μmoles/flask		*cpm/μmole*			*μg/ml cells*			*μg/ml cells*
1.6		1.28×10^6	0.3	0	108	1.7	0.2	109
8.2		2.50×10^5	0.5	0	110	2.0	0.3	110
41.0		5.00×10^4	1.0	0	108			108
206.0		9.90×10^3	1.7	0	111	8.2	1.2	111
	1.68	1.15×10^6	11.2	0.2	99	54.0	5.0	118
	Before incubation				119			106

* Incubation for 4 hours at 37° C.

† Each flask contained 10 ml washed erythrocytes (0.02% leukocytes) or washed reticulocytes (89% reticulocytes, 0.34% leukocytes) and substrates as described in the text.

‡ Relative specific activity = (counts per minute per μmole of product/counts per minute per μmole of precursor) × 100.

§ PN = pyridine nucleotides.

crease in the labeling of the TPN of reticulocytes paralleled the increased labeling of the DPN, but the RSA of the TPN was always about one-sixth that of the DPN. Net synthesis of total oxidized pyridine nucleotides in mature erythrocytes and in reticulocytes was not observed with any concentration of nicotinic acid.

Incorporation of nicotinamide. Experiments were performed with reticulocytes and erythrocytes of rabbits in which the nicotinic acid-7-C^{14} in the incubation medium was replaced by an equimolar amount of nicotinamide-7-C^{14} (Table II). These data indicate that there was a thirty-fold greater incorporation of nicotinamide into DPN than occurred with radioactive nicotinic acid. As with nicotinic acid, the incorporation of nicotinamide into the DPN of reticulocytes exceeded its incorporation into DPN of mature erythrocytes by about five times. Labeling of erythrocyte TPN was observed after incubation for 4 hours with nicotinamide, in contrast to the finding with nicotinic acid. The incorporation of radioactive nicotinamide into the TPN of reticulocytes exceeded its incorporation into the TPN of mature erythrocytes by about twenty-five times. Essentially identical results were obtained with nicotinamide in two additional experiments.

In agreement with Tulpule (26), net synthesis of total oxidized pyridine nucleotides upon incubation of rabbit erythrocytes with nicotinamide was not observed. Nor was there any evidence of significant net synthesis of total oxidized pyridine nucleotides in the rabbit reticulocytes incubated with nicotinamide.

DISCUSSION

A striking reduction in the ability to incorporate nicotinic acid and nicotinamide into the pyridine nucleotides is evident in mature erythrocytes when these cells are compared with reticulocytes obtained from rabbits with an acetylphenylhydrazine-induced hemolytic anemia. Since the concentrations of DPN and of TPN are essentially the same in reticulocytes as in erythrocytes (27), the findings described here indicate a more rapid turnover of these cofactors in reticulocytes.

It is unlikely that the sizes of the pools of the four forms of the pyridine nucleotides in erythrocytes and reticulocytes would have an appreciable effect on the pattern of isotopic labeling. Although DPNH and TPNH were not isolated in the present experiments, it is reasonable to assume that isotopic equilibration between the oxidized forms and their respective reduced forms occurred under the conditions of these experiments. Rapid equilibration of the reduced with the oxidized pyridine nucleotides has been shown to occur in rat liver *in vivo* (28).

Several reasons may be advanced to explain the decline in the ability of erythrocytes to incorporate nicotinic acid and nicotinamide into DPN as maturation proceeds from the reticulocyte stage. A decrease in the activity of one or more of the enzymes involved in the biosynthesis of DPN must be considered. Such an alteration would be analogous to the decline in the activity of other enzymes as mammalian erythrocytes mature and age. Another possibility is diminished availability of substrates for the biosynthetic enzymes. Since ATP and phosphoribosylpyrophosphate are required in all of the known pathways for the formation of DPN (29), the availability of these compounds may become limiting. A third possibility is that continued formation of DPN is inhibited by products of the biosynthetic reactions. For example, the activity of inorganic pyrophosphatase of rabbit reticulocytes appears to exceed greatly the activity in mature erythrocytes (30). Decreased ability to remove inorganic pyrophosphate, a product of the biosynthesis of DPN (29), might impair continued synthesis of the pyridine nucleotides (31). It is not possible, from the data presented here, to determine whether decreased activity of enzymes directly involved in biosynthesis, availability of substrates, or accumulation of reaction products is the critical process that leads to the decline in the ability to incorporate nicotinic acid and nicotinamide into DPN.

The incorporation of nicotinic acid and nicotinamide into TPN was much slower than into DPN in both erythrocytes and reticulocytes. Similarly, the incorporation of adenine-8-C^{14} into DPN was greater than into the TPN of human blood *in vitro* (32). Since the concentration of DPN in rabbit blood is much greater than that of TPN (33), the lower specific activity of the TPN cannot be attributed to the relative amounts of the pyridine nucleotides. A decline in the activity of DPN-kinase (29), or decreased avail-

ability of ATP or other cofactors may be responsible for the greater labeling of TPN in reticulocytes than in mature erythrocytes.

Alterations in the activities of nucleotidases, enzymes that hydrolyze DPN and TPN at the nicotinamide-ribose linkage and that can effect an exchange of the nicotinamide moiety (29), might be responsible for the differences observed between reticulocytes and mature erythrocytes. However, the activities of nucleotidases did not differ greatly when reticulocytes and erythrocytes of rabbits were compared (10, 34). It seems unlikely, therefore, that differences in nucleotidases alone could explain the greater incorporation of labeled precursors into DPN and TPN of reticulocytes.

Of considerable interest is the finding that nicotinamide-7-C^{14} was incorporated into the pyridine nucleotides of erythrocytes and reticulocytes to a far greater extent than was nicotinic acid-7-C^{14}. The currently accepted major pathway for the biosynthesis of DPN in human erythrocytes and in rat and mouse liver is thought to involve the intermediates, nicotinic acid mononucleotide and the nicotinic acid analogue of DPN (22, 29). A number of pathways by which nicotinamide may be incorporated into DPN have been described, but the extent of their contribution to the net synthesis of DPN remains unknown (29). It was suggested that nicotinamide may be utilized for pyridine nucleotide biosynthesis after deamidation at the free base, nucleoside, or mononucleotide level (35), and there is evidence for deamidation of nicotinamide mononucleotide by mouse liver (36). The observed difference in the incorporation of the two labeled precursors into the pyridine nucleotides of rabbit erythrocytes could be explained by rapid, preferential deamidation of nicotinamide riboside or nicotinamide mononucleotide before incorporation into DPN. It is of interest that the ratio of nicotinamide to nicotinic acid incorporation into DPN reported to occur with Ehrlich's ascites tumor cells *in vitro* (37) was similar to the ratio observed with erythrocytes and reticulocytes of rabbits. In contrast, the incorporation of nicotinic acid into the DPN of human erythrocytes exceeded that of nicotinamide by five to seven times (38). Thus, there appears to be a striking species and tissue specificity for the relative incorporation of nicotinic acid and nicotinamide into DPN. The greater incorporation of nicotinamide than of nicotinic acid observed in the present experiments might have resulted from greater permeability to the amide than to the free acid. Several investigations (39, 40), however, have indicated that the permeability of human erythrocytes to the two compounds is essentially equal. Another possible explanation for the apparent preferential incorporation of nicotinamide might depend upon the activity of nucleotidases that would favor incorporation of nicotinamide rather than nicotinic acid by an exchange reaction (29). This last possibility is supported by the failure to demonstrate net synthesis of total oxidized pyridine nucleotides in either reticulocytes or erythrocytes of rabbits.

SUMMARY

Radiocarbon-labeled nicotinic acid and nicotinamide are incorporated into the DPN and TPN of erythrocytes and reticulocytes of rabbits upon incubation of these cells *in vitro*. The incorporation of nicotinamide greatly exceeded the incorporation of nicotinic acid, at comparable concentrations, during 4 hours of incubation. The incorporation of both nicotinamide and nicotinic acid into the DPN of reticulocytes obtained from acetylphenylhydrazine-treated rabbits was four to five times greater than the incorporation into the DPN of mature erythrocytes. The labeling of the TPN of these reticulocytes was also much greater than the labeling of the TPN of erythrocytes. It would appear, therefore, that the ability to incorporate nicotinic acid and nicotinamide into DPN and TPN declines when rabbit reticulocytes mature to become nonreticulated erythrocytes. The precise mechanism that determines the diminished capacity to incorporate these precursors into the pyridine nucleotides during maturation remains to be defined.

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