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Pyridine Nucleotides in Erythrocyte Metabolism *

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In recent years investigations of erythrocyte metabolism have been concerned with the interrelationships of enzymes, coenzymes, substrates, cofactors, and pH, *inter alia* (1–8). Although data have accumulated on these various aspects of the metabolism of normal and pathological erythrocytes, little direct information has been available heretofore on pyridine nucleotide coenzymes, despite their importance in both the glycolytic and the hexose monophosphate shunt pathways. The availability of these compounds in the blood of immature subjects is of particular interest because of certain abnormalities in the erythrocytes of full-term newborn and prematurely born infants. These abnormalities, which include decreased survival of ^{51}Cr-labeled erythrocytes in premature infants (9, 10) and increased susceptibility to Heinz body formation (11) and to methemoglobinemia (12–15) in both full-term and premature infants, as well as the occurrence of unexplained hemolytic anemias, suggest the possibility of transient biochemical defects in the erythrocytes of these young subjects. Although the activities of the pyridine nucleotide-dependent enzymes in both the glycolytic and shunt pathways are increased in the erythrocytes of young infants (16, 17), the levels of the oxidized and reduced coenzymes themselves have not been reported.

In the present study the concentrations of DPN,[1]

DPNH, TPN, and TPNH have been determined in the blood of healthy human adults and premature infants and in cord blood by the sensitive technique of enzymatic cycling (18). Reported also are correlative studies to evaluate the response of oxidized and reduced pyridine nucleotides as well as pyridine nucleotide-requiring enzymes (PGD, LDH, G-6-PD, 6-PGD, GR, and Hb[3]R) to *in vitro* preservation of blood at 4° C in ACD, ACD-adenine, and ACD-inosine.

Methods

Subjects. The studies to be presented are a compilation of experiments performed independently in two laboratories, one in New York and the other in Seattle. Studies on adult human erythrocytes were undertaken in both laboratories. The samples from adult subjects both in New York and Seattle were obtained in each city from healthy hospital and laboratory personnel or blood donors and were studied only in the respective laboratories. Studies on erythrocytes from premature infants were performed in New York on infants selected from the premature nursery of the Bronx Municipal Hospital Center. Selection was made by exclusion of infants with recognized disease as well as infants receiving medications other than routine vitamin supplements. The premature infants ranged in age from 1 to 30 days and in weight from 1,240 to 2,260 g. The remainder of the studies were performed in Seattle. Umbilical cord blood was obtained at birth, immediately after the cord was cut, from full-term infants of known normal patients in the obstetrical service of Maynard Hospital in Seattle.

Materials. Materials used were obtained commercially.[2]

methemoglobin reductase; and ACD, acid-citrate-dextrose (National Institutes of Health, formula A).

[2] DPN was obtained from Pabst Laboratories, Milwaukee, Wis., or from Sigma Chemical Co., St. Louis, Mo.; TPN, TPNH, DPNH, glucose-6-phosphate, adenine sulfate, and protamine sulfate from Sigma Chemical Co.; crystalline bovine serum albumin from Armour and Co., Kankakee, Ill.; α-ketoglutarate, 6-PGD, G-6-PD, glutamic dehydrogenase, and rabbit muscle LDH from Boehringer and Sons, Mannheim, Germany; calcium lactate from Calbiochem, Los Angeles, Calif.; beef heart LDH from Worthington Biochemical Corp., Freehold, N. J.; EDTA from J. T. Baker Chemical Co., Phillips-

Procedures. Blood was collected by venipuncture in ACD solution or heparin (2 mg per 100 ml of blood) or was used directly from a free flowing deep capillary puncture obtained from the finger tip in adults and from the heel in premature infants. Four aliquots of 10 μl each was sufficient for the measurement of the oxidized and reduced pyridine nucleotides.

For the studies on erythrocyte preservation, blood was collected in ACD and separated immediately into three aliquots of 100 ml each. The aliquots were supplemented with 10 ml of 0.9% NaCl (control), adenine sulfate (0.9 to 1.0 μmole adenine per ml of blood), or inosine (10.0 to 10.2 μmoles per ml). The blood was stored at 4° C in Cutter plastic bags.

DPN, DPNH, TPN, and TPNH were determined in both laboratories by the enzymatic cycling method of Lowry, Passonneau, Schulz, and Rock (18). The nucleotides were used at concentrations well below their Michaelis constants during cycling. The reaction rates were proportional to nucleotide concentrations over the range of 2×10^{-9} to 1×10^{-8} mole per L for TPN and TPNH and 5×10^{-10} to 5×10^{-9} mole per L for DPN and DPNH. In the Seattle laboratory the final fluorometric measurements, made in a volume of 2.30 ml for DPN and DPNH and 2.50 ml for TPN and TPNH, were performed with the use of the G. K. Turner fluorometer with primary Corning 5860, secondary Turner 3, and Turner 48 filters. In the New York laboratory the final volumes employed were 1.22 ml for DPN and DPNH and 1.11 ml for TPN and TPNH, and the fluorometric measurements were performed with a Farrand spectrophotofluorometer with primary Corning 737 and secondary Corning 372 filters. For measurements of DPN and DPNH the wavelength of the exciting monochromator was 370 mμ and of the analyzing monochromator 475 mμ. For TPN and TPNH the wavelengths were 350 and 480 mμ for the two monochromators.

Purification of 6-PGD from rat liver was performed according to the procedure of Lowry and associates (18) with the following modifications. Nucleic acids were precipitated directly from the homogenate by the addition of 0.3 vol of 2% protamine sulfate. After the first dialysis, the dialyzed preparation was added to 1 vol of DEAE-cellulose suspension, prepared as described previously (2), and mixed for 15 minutes. About 40% of the G-6-PD remains on the adsorbent. The unadsorbed fraction, collected by centrifugation at 1,900 g in the International refrigerated centrifuge model PR-2, was treated with ammonium sulfate according to the original procedure. After the second dialysis, treatment of the dialyzed preparation with DEAE-cellulose was substituted for the series of calcium phosphate gel treatments, and the 6-PGD in the unadsorbed fraction was purified further as described originally. The above modifications

served to expedite the preparation and insure more consistent results.

Leukocytes from 90 ml of pooled cord blood were isolated by a technique described previously (19). Hemoglobin was determined as cyanmethemoglobin (20) and plasma hemoglobin as pyridine hemochromogen (21). Assays for the following enzymes were performed on hemolysates (1, 2) essentially according to the published methods: G-6-PD (22), 6-PGD (23), LDH (24), PGD (1), GR (25), and Hb^{8}R (26).

Results

Reliability of method. The stability of DPN, DPNH, TPN, and TPNH in blood samples collected in ACD and maintained thereafter at 4° C was tested at 5, 15, and 50 minutes and 1, 12, and 24 hours after collection. A variable decline, no greater than 15%, in one or more of the nucleotides was encountered in blood samples obtained from adults after 1 hour of storage. In samples from premature infants, there was no difference from adults in the stability of TPN and TPNH. However, 1 hour after collection there were losses as great as 40% in both DPN and DPNH in samples from premature infants. Once the aliquots of blood were diluted in the appropriate acid or alkaline solutions, the levels of all four nucleotides from all subjects remained stable at 4° C in the refrigerator, but lost variable amounts of activity at the lower temperature of the laboratory ice bucket. These latter observations on the stability of dilute solutions of endogenous pyridine nucleotides are comparable to the findings of Lowry, Passonneau, and Rock (27) that dilute standard solutions of pyridine nucleotides are more stable at 4° C than at −20° C.

A comparison of values obtained from analyses of whole blood and from isolated, washed red cells showed that the process of washing the cells resulted in variable losses, up to 15% of the nucleotides.

The recovery of pyridine nucleotides added to diluted blood samples just before heating was 97% for DPN and TPN, 98% for DPNH, and 96% for TPNH. Duplicate determinations of a particular blood sample agreed to within 5%.

Mean levels of pyridine nucleotides in the erythrocytes of healthy adults obtained and determined independently in Seattle and New York differed at the most by 12% for TPN and as little as 2% for TPNH (cf. Table I).

burg, N. J.; DEAE-cellulose from Eastman Kodak Co., Rochester, N. Y.; dextran from Pharmacia Laboratories, Inc., New Market, N. J.; inosine from Schwarz Bio-Research, Mt. Vernon, N. Y.; and sodium heparin (10 mg per ml) from Invernex Corp., San Francisco, Calif.

TABLE I

Levels of pyridine nucleotides in erythrocytes from adults, premature infants, and cord blood

Source of blood	DPN	DPNH	TPN	TPNH	$\dfrac{DPN +}{DPNH}$	$\dfrac{TPN +}{TPNH}$	$\dfrac{DPN}{DPNH}$	$\dfrac{TPN}{TPNH}$	$\dfrac{DPN + DPNH}{TPN + TPNH}$
Adult subjects				*mµmoles/g hemoglobin*					
New York	188 ± 6.1* (155–240)† (14)‡	142 ± 6.1 (97–204) (13)	121 ± 4.9 (89–161) (30)	87 ± 5.8 (54–129) (13)	330	208	1.3	1.4	1.6
Seattle	205 ± 7.8 (162–248) (19)	153 ± 9.3 (99–217) (19)	106 ± 5.0 (73–133) (19)	85 ± 4.3 (55–104) (19)	358	191	1.3	1.2	1.9
Combined	198 ± 7.1 (155–248) (33)	149 ± 8.0 (97–217) (32)	115 ± 4.9 (73–161) (49)	86 ± 4.9 (54–129) (32)	347	201	1.3	1.3	1.7
Cord	240 ± 9.2 (179–295) (15)	173 ± 5.5 (136–210) (15)	126 ± 3.4 (108–150) (15)	90 ± 4.2 (65–118) (15)	413	216	1.4	1.4	1.9
Premature infants	205 ± 7.7 (133–299) (17)	134 ± 7.8 (105–221) (17)	127 ± 5.0 (102–167) (30)	80 ± 4.9 (51–103) (22)	339	207	1.5	1.6	1.6

* Mean value ± standard error of mean.
† Range.
‡ No. of subjects.

Pyridine nucleotide levels of erythrocytes from adults, premature infants, and cord blood. In Table I is presented a summary of the erythrocyte pyridine nucleotide levels from the three groups of subjects. The mean values do not differ appreciably among the groups. No deficiency could be demonstrated in the erythrocytes of either the full-term newborn (cord blood) or premature infants. Within each group the ratio of oxidized to reduced nucleotide was the same for both DPN and TPN. Although there was a decrease in this ratio concomitant with increasing maturity from premature to cord to adult blood, the significance of the widest difference, between premature infants and adults, is questionable $(0.1 > p > 0.05$, determined by Student's t test). In Table II the levels encountered in adults in the present study are further compared with results reported by others using a variety of methods.

Leukocyte contribution to pyridine nucleotide levels in blood. Although the values for pyridine nucleotides in the erythrocyte are derived from

TABLE II

Pyridine nucleotides in blood*: comparison of levels obtained from adults in the present study with those reported in the literature

Author	Reference no.	Species	DPN	DPNH	TPN	TPNH
			moles ×10⁻⁵		*moles ×10⁻⁶*	
Present study*		Man	3.0	2.2	1.65	1.25
Szeinberg and Pipano	28	Man	2.6		1.92	
Barnett	29	Man	4.5–5.5		1.2–1.6	
Schrier, Kellermeyer, and Alving	30	Man	5.3		3.4	
Bishop, Rankine, and Talbott	31	Man	2.98–3.24		1.12–1.16	
Mills and Summers	32	Man	2.6		1.5	
Bonsignore, Fornaini, Segni, and Fantoni	33	Man	6.7		2.0	
Shafer and Bartlett	34	Man	3.5		1.3	
Oski and Diamond	35	Man	3.2		0.85	
Löhr and Waller	36	Man	3.3	0.46	1.16	1.6
Brown and Clarke	37	Man				0.4–6.8
Jaffé and Neumann	38	Man	2.9–3.3		1.37–1.55	
Lowry, Passonneau, Schulz, and Rock	18	Rat	11.0	8.7	1.38	1.15
Glock and McLean	39	Rat	8.3	5.5	0.74	0.41
		Rabbit	5.0	0.6	0.41	0.27

* Calculated on basis of 15 g hemoglobin per 100 ml blood.

TABLE III

Contribution of leukocytes to levels of pyridine nucleotides in whole blood*

	Adult blood		Cord blood	
Nucleotide	Erythro-cytes	Leuko-cytes	Erythro-cytes	Leuko-cytes
	mμmoles/ml whole blood			
DPN	25.8†	0.21‡	31.2†	0.24§
DPNH	19.3	0.16	22.5	0.04
TPN	13.4	0.05	16.4	0.07
TPNH	10.7	0.16	11.7	0.21

* Erythrocyte values in adult and cord blood and leukocyte values in cord blood are derived from data obtained in the present study using the technique of enzymatic cycling. The leukocyte values in adult blood are calculated from the data of Silber, Gabrio, and Huennekens (19) obtained by a different method.
† Calculated by mean nucleotide levels given in Table I and mean hemoglobin levels of 12.6 and 13.0 g hemoglobin per 100 ml acid-citrate-dextrose (ACD) blood for adult and cord blood, respectively.
‡ Calculated assuming an average leukocyte count in ACD blood of 6,500 per mm³.
§ Leukocyte count in cord blood sample = 13,300 per mm³.

analyses of whole blood, the data given in Table III indicate that this is justified. The figures given for erythrocytes are those obtained on whole blood, whereas those for leukocytes represent analyses made on isolated leukocyte preparations. It is apparent that the leukocyte contribution is negligible, i.e., less than 1% of the determined values for DPN, DPNH, and TPN and about 1.5% of the values for TPNH in both adult and cord blood. If the total pyridine nucleotide in the formed elements of the blood is calculated on a cellular basis (cell count), the values are 190 mμmoles per 10^{10} erythrocytes and 890 mμmoles per 10^{10} leukocytes.

In vitro storage of blood: pyridine nucleotides. Three experiments were performed in which blood was collected in ACD, supplemented with adenine or inosine, and analyzed at weekly intervals for 48 days during storage at 4° C. The results

were essentially the same in all three experiments. In Table IV are shown data from a representative experiment in which the additions at the beginning of storage were 0.9 μmole of adenine or 10.0 μmoles of inosine per ml of ACD blood. Although there were no remarkable changes in pyridine nucleotide levels throughout the metabolic stress of storage, there was a small net loss of diphosphopyridine nucleotide in the control sample. The ratio of the oxidized to reduced forms decreased in all blood samples as a consequence of the slight fall in the levels of DPN and TPN and a compensatory slight increase in DPNH and TPNH. The significance of these small changes is not apparent.

In vitro storage of blood: pyridine nucleotide-requiring enzymes. Concomitant with the relative stability of the pyridine nucleotides during storage of blood at 4° C are the essentially unchanged activities of several pyridine nucleotide-dependent enzymes in the erythrocyte (cf. Table IV).

Discussion

The method of enzymatic cycling (18) has provided a precise and highly reproducible means of measuring each of the four pyridine nucleotide forms in micro quantities of blood. The concordance of data obtained from independent samples determined in two different laboratories is testimony to this fact. Comparative data obtained from isolated preparations of leukocytes indicate that the quantities measured in whole blood may be equated with erythrocyte pyridine nucleotide levels.

The concentrations of both DPN and TPN in adult blood shown in Table II are similar to those

TABLE IV

Pyridine nucleotides and pyridine nucleotide-requiring enzymes in the erythrocyte during in vitro storage at 4° C*

	Pyridine nucleotides				Enzymes						Plasma hemo-globin
Days stored	DPN	DPNH	TPN	TPNH	PGD	LDH	G-6-PD	6-PGD	GR	Hb³R	
	mμmoles/g hemoglobin				*μmoles substrate converted/hour/g hemoglobin*						*mg hemo-globin/ 100 ml ACD plasma*
0	194	150	114	92	828	1,470	87	92	31	10	8
48											
Control	161	145	106	93	805	1,485	85	90	34	11	161
Adenine	175	161	99	102	772	1,453	111	95	33	11	141
Inosine	173	163	100	100	767	1,378	105	82	29	10	120

* PGD = 3-phosphoglyceraldehyde dehydrogenase, LDH = lactic dehydrogenase, G-6-PD = glucose-6-phosphate dehydrogenase, 6-PGD = 6-phosphogluconic dehydrogenase, GR = glutathione reductase, and Hb³R = TPNH-methemoglobin reductase.

reported by a number of investigators using different analytical techniques. Few comparative data are available for reduced pyridine nucleotides. In the present study, DPNH content is about five times greater and the TPNH level is only 80% of the respective values reported by Löhr and Waller (36), who employed a different method for analysis. With the use of a modification of the present method of enzymatic cycling for the TPNH assay, Brown and Clarke (37) have reported a mean concentration of TPNH that is 2½ times greater than the present value. However, their data were obtained on 23 clinic patients without an attempt to correlate clinical diagnoses, and the authors state that the wide range of values might indicate the presence of abnormal specimens. This fact, coupled with the methodological difference whereby the depletion of glucose-6-phosphate in the enzymatic cycling mixture was measured, may contribute to the variance of data as compared with the present study.

There is almost twice as much total DPN as TPN in the human erythrocyte. This ratio is similar to the distribution in a number of other tissues reported by Lowry and his associates (18) and by Burch, Lowry, and Von Dippe (40). In the steady state condition the quantity of the oxidized forms of both DPN and TPN exceeds the reduced forms. Furthermore, the quantity of oxidized TPN appears adequate for the dehydrogenase reactions of the hexose monophosphate shunt pathway. The TPN level of 10^{-5} mole per L is an order of magnitude higher than the K_m for TPN in the G-6-PD reaction in human erythrocytes (41, 42). Thus additional investigation appears to be indicated to reveal why glycolysis predominates in the erythrocyte.

The present studies provide no evidence for pyridine nucleotide deficiency in the erythrocytes of full-term newborn or premature infants. This observation is of interest in view of abnormalities characteristic of the erythrocytes of young infants. Limited availability of TPN in particular has been considered a possible explanation for the increased susceptibility to Heinz body formation and drug-induced hemolysis in newborn infants (43). It should be emphasized, therefore, that measurements in this study have been obtained in the absence of oxidative stress to the erythrocytes. Since the rate of erythrocyte glycolysis is greatly

increased in newborn infants, particularly in the presence of an oxidizing agent (44, 45), the occurrence under stress of a relative deficiency of one or more of the pyridine nucleotides has not been ruled out. The further possibility that oxidants may have a direct effect upon erythrocyte pyridine nucleotide levels is considered in detail elsewhere (46).

Stability studies demonstrating losses of DPN and DPNH as great as 40% when whole blood from premature infants is maintained at 4° C for 1 hour suggest that the erythrocytes of these subjects have increased activity of the enzyme that hydrolyzes the DPN. Although this hydrolytic activity ceases upon dilution of the blood in acid or base, it is possible that unappreciated losses may be sustained during the 1 to 2 minutes often involved in collecting blood from premature infants. Thus the *in vivo* levels of the DPN in the erythrocytes of premature infants may be somewhat higher than those reported here. The finding of slightly higher levels of DPN and DPNH in cord blood from full-term infants and the stability of these compounds in cord blood at 4° C indicates that the presumed increased activity of DPNase in the premature is no longer evident in the erythrocytes of full-term newborn infants.

During the *in vitro* preservation of blood at 4° C in ACD, numerous physical and chemical changes occur in the erythrocyte. Supplementation of the blood with adenine (47), nucleosides (inosine or adenosine) (48–50), or both (51) allays many of the deteriorative changes. The present evidence indicates, however, that pyridine nucleotides and pyridine nucleotide-dependent enzymes are maintained at approximately their steady state levels throughout storage with or without additives. These findings are of interest in light of the evidence of Jaffé and Neumann that stored erythrocytes have a diminished capacity to synthesize DPN from nicotinic acid as well as incorporate nicotinic acid or nicotinamide into DPN and TPN in comparison with fresh cells (38). Further, Jaffé and Neumann found that although inosine is inhibitory to these processes in fresh blood, the nucleoside could effect, in part, a restoration of the ability of stored cells to carry out these reactions (38). Since the pyridine nucleotide levels in the present study remained stable throughout storage despite the decline in the cell's

pertinent synthetic capacity, it might be concluded that the enzymes that hydrolyze pyridine nucleotides in erythrocytes of adults are inactive during storage at 4° C. Quite different results in the levels of these nucleotides and enzymes can be obtained if the temperature of storage is increased to 20° C. Löhr and Waller (4) found that DPN and TPN declined to about 20% of their original levels and PGD, LDH, G-6-PD, and Hb^{3}R to about 10 to 40% of their original activities after 7 days of storage at 20° C. The latter data serve to emphasize that experiments performed on blood stored at elevated temperatures are not comparable to those carried out at the standard 4° C.

Summary

The concentrations of oxidized and reduced diphosphopyridine nucleotide and triphosphopyridine nucleotide have been determined and are essentially the same in erythrocytes of adults, premature infants, and cord blood. There is almost twice as much total DPN as total TPN, and in the steady state both nucleotides exist predominantly in the oxidized form. Comparative data from adult and cord leukocytes indicate that measurements of whole blood are valid indexes of the pyridine nucleotide content of erythrocytes.

During the *in vitro* preservation of blood at 4° C in acid-citrate-dextrose solution or with added adenine or inosine, the levels of oxidized and reduced pyridine nucleotide remain fairly constant as do the activities of pyridine nucleotide-requiring enzymes, phosphoglyceraldehyde dehydrogenase, lactic dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, glutathione reductase, and TPNH-methemoglobin reductase.

Acknowledgments

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