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URIDINE DIPHOSPHATE GALACTOSE 4-EPIMERASE IN HUMAN AND OTHER MAMMALIAN HEMOLYSATES

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SUMMARY

1. In contrast to the requirement for exogenous NAD^+ in the assay of UDP-galactose 4-epimerase (UDPgalactose epimerase, EC 5.1.3.2), in hemolysates from adults, substantial epimerase activity can be demonstrated in hemolysates from newborn infants without addition of NAD^+. It has been shown that erythrocytes of newborns are deficient in stromal NAD nucleosidase; and that a considerable portion of intracellular NAD^+ remains in the hemolysate. In the preparation of hemolysates of adults, NAD^+ of intracellular origin is destroyed by activity of stromal NAD nucleosidase.

2. Upon starch gel electrophoresis, two distinctly separate activity bands were found for epimerase in hemolysates from newborn infants and one distinct band, of different mobility, for the enzyme in hemolysates of adults. In the presence of NAD^+ in the gel, electrophoresis of hemolysates of adults resulted in the two-banded epimerase pattern found for newborns. It is presumed that an NAD^+-dependent structural change in the enzyme is involved.

3. Two-banded electrophoretic patterns for epimerase were found in hemolysates of a number of mammals other than man, each set having a different electrophoretic mobility from that of any other.

INTRODUCTION

Exogenous NAD^+ is required for demonstrating UDPgalactose 4-epimerase (EC 5.1.3.2) activity in hemolysates of normal adults but not of newborn infants[1,2]. The present report provides an explanation for the latter. Erythrocytes of newborn infants have been found to be deficient in stromal NAD nucleosidase activity, and endogenous NAD^+ therefore remains in hemolysates.

Upon starch gel electrophoresis, epimerase in hemolysates from newborn infants has been shown to migrate in two distinct bands, that from adults in a single band of different mobility[3,4]. The difference now has been demonstrated to be a

function of the NAD^+ concentration present, rather than a qualitative difference dependent upon age of the individual.

MATERIALS AND METHODS

Hemolysates were prepared from erythrocytes as previously described[5]. Stroma preparations were made according to the procedure of Carson *et al.*[6].

UDPglucose, NAD^+, UDPglucose dehydrogenase, alcohol dehydrogenase and NAD nucleosidase *(Neurospora)* were obtained from the Sigma Chemical Company. UDPgalactose was from Calbiochem.

UDPgalactose 4-epimerase was assayed in two steps as previously described[2]. UDPgalactose was converted to UDPglucose, and then the amount of UDPglucose formed was determined by coupling to NAD^+ reduction in the presence of UDPglucose dehydrogenase. NAD nucleosidase was assayed according to the procedure described in a previous publication[7]. Activity is expressed as μmoles of NAD^+ hydrolyzed/h per ml red blood cells.

The NAD^+ concentration in whole blood or in 50% hemolysates was determined by the following procedure.

Extraction of NAD^+

To 1 ml of whole blood or 1 ml of 50% hemolysate, 4 ml of 0.3 M cold $HClO_4$ were added. The suspension was mixed by inversion, allowed to stand in an ice bath for 10 min and then centrifuged at 12 000 $\times$ *g* at 2 °C for 30 min. The supernatant was poured into a 12-ml centrifuge tube. To the supernatant 0.1 ml of 5 M K_2CO_3 and 0.2 ml of 2.5 M KCl were added. After mixing with a cyclomixer, followed by standing in an ice bath for 1 h, the mixture was centrifuged at 3000 $\times$ *g* for 10 min. A 1-ml aliquot of the supernatant was used for NAD^+ determination. To another aliquot of 1 ml, serving as a control, 50 μl of NAD nucleosidase (5 mg/3 ml water) were added, and the mixture was incubated at 37 °C for 60 min.

Determination of NAD^+

NAD^+ was determined fluorometrically according to Ciotti and Kaplan[8], in the presence of methyl ethyl ketone. Experiments with known amounts of NAD^+ gave a recovery of 80% or more.

Horizontal starch gel electrophoresis was carried out by conventional techniques. The buffer system was the same as that used for galactose-1-phosphate uridyl transferase[9], but the electrophoresis was carried out at a lower voltage because of the greater mobility of epimerase. At the end of the run, the gels were sliced in half, and the cut surfaces were overlayed with a reagent gel. The detection system depended upon the conversion of UDPgalactose to UDPglucose by epimerase, followed by coupling to reduction of NAD^+ in the oxidation of UDPglucose by UDPglucose dehydrogenase. The reagent for two trays consisted of 0.7 ml NAD^+ (7 mg/ml), 0.3 ml UDPgalactose (3 mg/ml), 0.1 ml 2-mercaptoethanol (0.3 M), 0.2 ml of UDPglucose dehydrogenase (Sigma, 5000 units/ml) and water to a total volume of 3.8 ml. Immediately before use, an equal volume of a starch solution was added (2.7 g of starch heated in 40 ml of 0.125 M glycine buffer, pH 8.7, kept at 40 °C). An appropriate control (omission of UDPgalactose) also was employed. After pouring and

spreading the reagent gel mixture on the surface, the starch gel was incubated in a moist chamber at 37 °C for 1–4 h. Epimerase was detected by the appearance under long wave ultraviolet light of fluorescent bands of NADH.

RESULTS

Epimerase assay

In Table I is presented data for a group of 36 newborn infants. In each case, hemolysate UDPgalactose 4-epimerase activity was demonstrated without addition of NAD^+ in the assay. Among individuals, this activity ranged from 40 to 100% of that with NAD^+ added. In a group of 37 normal adults epimerase activity was absent or very low without exogenous NAD^+. In the present study, as well as in a previous report, the mean value for epimerase activity in hemolysates from newborns was found to be significantly higher than that for adults[2]. Hemolysate epimerase activity decreases with age until the adult level is reached by the end of the first year of life (Fig. 1).

TABLE I

UDP GALACTOSE 4-EPIMERASE ACTIVITY IN HEMOLYSATES OF NORMAL NEWBORNS AND ADULTS

Incubation mixture consisted of 100 μmoles of glycine buffer, pH 8.7, 0.42 μmole of UDPgalactose with or without 1 μmole of NAD^+ as indicated, and 0.5 ml of freshly prepared 50% hemolysate in a total volume of 0.8 ml. Incubation period is 30 min at 37 °C. Epimerase activity is expressed as μmoles substrate consumed/h per ml red blood cells.

Group	*Epimerase activity*	
	Without NAD^+	*With NAD^+*
Newborn infants (36)		
Mean	2.32	3.09
S.D.	0.74	0.55
Range	1.08–4.05	2.00–5.16
Adults (37)		
Mean	0.05	1.54
S.D.	0.07	0.51
Range	0–0.21	0.46–2.43

Kirkman and Maxwell[10] found that preparation of hemolysates in the presence of nicotinamide, a known NAD nucleosidase inhibitor, results in demonstrable epimerase activity without the addition of NAD^+. This suggested to us that erythrocytes of newborn infants might be deficient in NAD nucleosidase activity[2].

When hemolysate NAD nucleosidase activity was measured in individuals of different ages, it was found to be at zero or very low levels in newborn infants, to increase with age and to reach the adult level by the end of the first year of life (Fig. 2). The mean activity for adults is about six times that for the newborn group. This increase corresponds inversely to the decrease with age of epimerase activity independent of exogenous NAD^+.

NAD nucleosidase activity in hemolysates from human erythrocytes has been shown to be localized in the stroma[11]. The addition of stroma from erythrocytes of

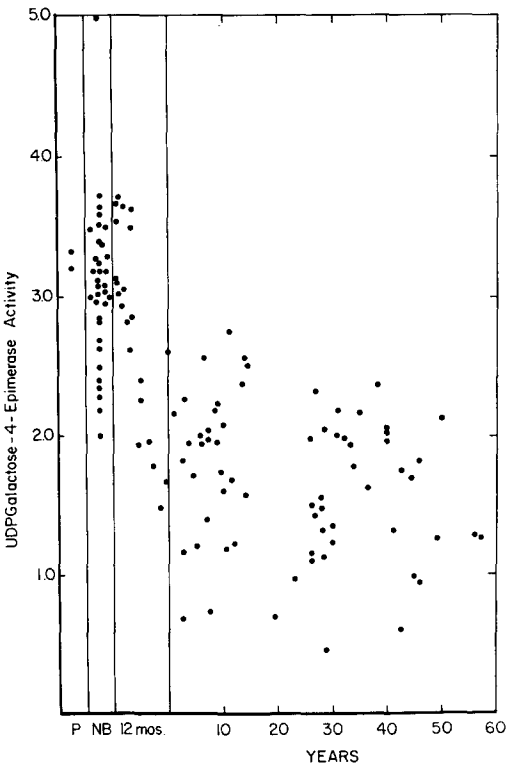

Fig. 1. Hemolysate UDPgalactose 4-epimerase activity in relation to age (P, premature; NB newborn period). NAD^+ added to all incubations. Each point represents the value for one individual.

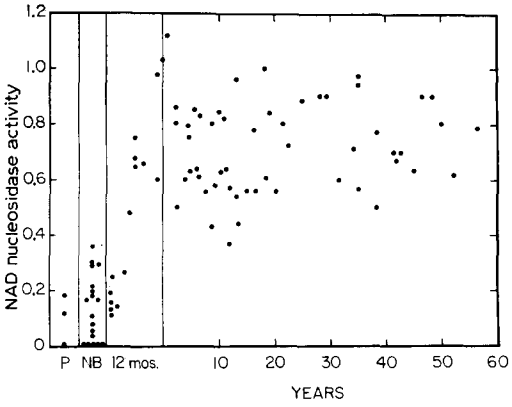

Fig. 2. Hemolysate NAD nucleosidase activity in relation to age (P, premature; NB, newborn period). Each point represents the value for one individual.

an adult, or addition of a commercial NAD nucleosidase preparation, to hemolysates of newborns results in loss of epimerase activity unless NAD^+ is present (Table II).

The findings shown in Fig. 2 suggest that it should be possible to demonstrate a marked difference in NAD^+ concentration between hemolysates of erythrocytes of

TABLE II

EFFECT OF STROMAL AND *Neurospora* NAD NUCLEOSIDASE ON UDPGALACTOSE 4-EPIMERASE ACTIVITY OF NEWBORN HEMOLYSATES

Experimental conditions similar to those for Table I. Stroma prepared from erythrocytes of a normal adult. In the preincubation experiments, UDPgalactose, and NAD^+ as indicated, were added after the preincubation period.

Newborn	*Conditions*	*Epimerase activity*	
		Without NAD^+	*With NAD^+*
1	Without stroma	1.19	1.52
	With stroma	0	0.42
2	Without stroma	1.98	3.00
	With stroma	0.25	1.66
	30 min preincubation in buffer without stroma	1.19	2.73
	30 min preincubation in buffer with stroma	0.02	0.31
3	Without NAD nucleosidase	2.32	2.79
	With NAD nucleosidase *(Neurospora)*	0.28	0.26

newborn infants as compared to those from adults. Data are presented in Table III. The NAD^+ concentration in the hemolysates from newborns was essentially the same as in the intact cells, but the level in hemolysates from adults dropped to about 30% of that in whole blood. The difference was considered to reflect the effect of stromal NAD nucleosidase, since it has been reported that the NAD^+ concentration in whole blood, both of the newborn and of the adult, remains stable during 24 h storage at 4 °C (ref. 12).

TABLE III

COMPARISON OF MEAN NAD^+ CONCENTRATIONS IN WHOLE BLOOD AND IN CORRESPONDING HEMOLYSATES

Group	*NAD^+ concentration (μg/g hemoglobin)*	
	Whole blood	*Hemolysate*
Newborns (4)	129	144
Adults (5)	171	59

Further evidence was obtained by preparing hemolysates with and without nicotinamide, a known NAD nucleosidase inhibitor[10]. The results are summarized in Table IV. It is evident that NAD^+ need not be added in the epimerase assay of hemolysates from adults if nicotinamide is used in the preparation of the hemolysate.

Starch gel electrophoresis

On starch gel electrophoresis, two well-defined fluorescent bands of epimerase activity were observed with newborn hemolysates and one with adult hemolysates (Fig. 3). The single band for the normal adult is intermediate in position to the two bands for the newborn infant. In the absence of UDPgalactose in the incubation medium, no fluorescent areas could be detected. As the time of incubation with the

TABLE IV

EFFECT OF NICOTINAMIDE UPON MEAN EPIMERASE ACTIVITY

Hemolysate preparation	*Epimerase assay*	*Mean epimerase activity**	
		Newborns (10)	*Adults (12)*
Without nicotinamide	Without NAD	2.18	0.04
	NAD added	3.90	1.42
With nicotinamide**	Without NAD	4.33	1.93
	NAD added	4.05	1.73

* μmoles UDPgalactose converted/h per ml red blood cells (incubation period 10 min).
** Equal volume of 0.2 M nicotinamide.

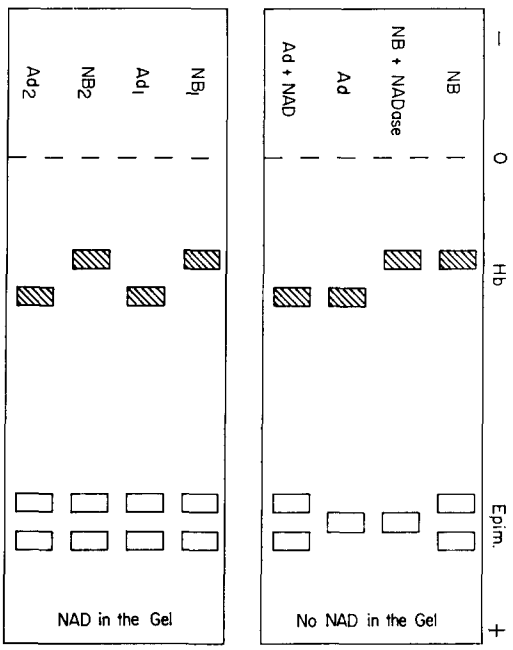

Fig. 3. Electrophoretic patterns of hemolysate epimerase in absence and in presence of NAD^+ in gel (NB, newborn; Ad, adult). Each sample applied as 50% hemolysate on Whatman 3MM filter paper (10 mm × 5 mm) into slot in gel. NADase, NAD nucleosidase.

reagent gel was extended, a faint band appeared behind the strongly fluorescing band for adult hemolysates.

The difference between the electrophoretic patterns is a function of the presence or absence of NAD^+. When a newborn hemolysate was preincubated for 1 h with a NAD nucleosidase preparation, or with stroma from erythrocytes of an adult, one band (in the adult position) was obtained. On the other hand, apparent conversion of the adult to the newborn pattern resulted either from: (1) the presence of NAD^+ in the starch gel at $1 \cdot 10^{-5}$ M; (2) incubation of adult hemolysates prior to electrophoresis for 30 min with $1 \cdot 10^{-3}$ M NAD^+.

TABLE V

COMPARISON BETWEEN UDPGALACTOSE 4-EPIMERASE AND NAD NUCLEOSIDASE ACTIVITIES ASSAYED IN MAMMALIAN HEMOLYSATES

Species	*NAD nucleosidase activity*	*Epimerase activity*	
		Without NAD+	*With NAD+*
Man			
Newborns	0–0.30	1.07–4.05	2.00–5.16
Adults	0.35–1.11	0–0.20	0.46–2.36
Calf	> 2.0	0	0
Rabbit	> 2.0	0	0
Cat	0	2.89	2.48
Mouse	0	2.11	2.27

The hemolysate epimerase studies were extended to other mammals (Table V). The same inverse relationship was found between hemolysate NAD nucleosidase activity and activity of epimerase in the absence of exogenous NAD^+. For the calf and the rabbit, the NAD nucleosidase activity was so high that epimerase could not be demonstrated even upon addition of NAD^+ to the assay or after preparation of the hemolysates with nicotinamide. However, upon electrophoresis, with NAD^+ present in the gel, epimerase activity could be demonstrated in both cases. When NAD nucleosidase activity was absent, as in the hemolysates of the cat and the mouse[13], the NAD^+-independent epimerase activity was found to be substantially the same as in the presence of NAD^+. Two-banded epimerase electrophoretic patterns were obtained for each of the four animals, differing in mobility from each other and from that for man.

DISCUSSION

The evidence presented indicated that sufficient endogenous NAD^+ remains in hemolysates of newborn infants to permit demonstration of epimerase activity without the addition of NAD^+. NAD nucleosidase activity is deficient or absent in hemolysates from newborn infants. In contrast, as erythrocytes from adults are lysed, stromal NAD nucleosidase rapidly destroys the NAD^+ which had been present in the cell.

The hemolysate epimerase of the adult requires addition of NAD^+ for demonstration of activity[1]. However, when nicotinamide, a known NAD nucleosidase inhibitor, is employed in the preparation of such hemolysates, addition of NAD^+ to the epimerase assay is no longer necessary[10].

In the presence of NAD^+ in gel electrophoresis, both adult and newborn hemolysate epimerase migrate similarly. Removal of NAD^+ from the hemolysate, and omitting it from the gel, changes the electrophoretic pattern, suggesting an NAD^+-dependent change in structure. The nature of this change remains to be explored. The sensitivity to NAD nucleosidase suggests that in the hemolysate enzyme, NAD^+ is not tightly bound. A requirement of exogenous NAD^+ for epimerase activity has been shown for calf liver and for homogenates of L-cells and of HeLa cells[14,15]. Epimerase preparations from yeast and from *Escherichia coli* do not require

exogenous NAD^+ for activity: in these preparations NAD^+ is present, tightly bound to the enzyme[16,17]. Evidence for the existence of monomer, dimer and tetramer forms of yeast epimerase under various conditions has been reported[18]. Epimerase prepared from *E. coli* appears to be a complex consisting of two identical polypeptides and one molecule of NAD^+ held together by noncovalent bonds[19]. The difference in requirement of NAD^+ for activity of mammalian epimerase suggests a different relationship with respect to structure than postulated for the yeast or the *E. coli* enzymes.

In the present study it was found that hemolysates from a number of mammals other than man contain an epimerase which migrates in a two-banded pattern on electrophoresis. Two electrophoretic forms of bovine mammary epimerase have been reported by Tsai *et al.*[20]. In that case, the pattern was influenced not only by its cofactor (NAD^+) but also by its substrate, UDPglucose.

An interesting observation in the present study has been that epimerase activity could be demonstrated by electrophoresis in hemolysates from rabbit and from calf even though activity could not be found by the conventional assay. In each case, the NAD nucleosidase activity of the hemolysate was high. The inability to demonstrate epimerase activity by the usual assay does not necessarily mean that the enzyme is not present.

Products of NAD nucleosidase activity may also be a factor. It has been shown in human hemolysates that progressive inactivation of 6-phosphogluconate dehydrogenase occurred in the presence of stromal NAD(P) nucleosidase, together with a change in electrophoretic pattern[21]. Similar results were obtained by the use of 2′-phosphoadenosine diphosphate ribose, a hydrolytic product of NADP.

The function of erythrocyte NAD nucleosidase is not known. Deficiency of erythrocyte NAD nucleosidase is not confined to newborn infants. It has been shown that reduced or absent erythrocyte NAD nucleosidase activity is not uncommon among adult Negroes and that the deficiency appears to be genetically determined[7]. Among the various species of animals studied, some do not have erythrocyte NAD nucleosidase activity (*e.g.*, cat, mouse).

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