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Enzymic basis of deranged foetal flavin-nucleotide metabolism consequent on immunoneutralization of maternal riboflavin carrier protein in the pregnant rat

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A comparison of the kinetic and other parameters of enzymes of flavin-nucleotide metabolism in the whole foetus *vis-à-vis* the maternal liver in the pregnant rat revealed relatively lower activities of foetal flavokinase and FAD pyrophosphorylase. Passive immunoneutralization of the maternal riboflavin carrier protein suppresses foetal FAD pyrophosphorylase rather selectively. Additionally, although the activities of foetal nucleotide pyrophosphatase and FMN phosphatase were unchanged owing to immunoneutralization, higher activities of these enzymes in the whole foetus as compared with the maternal liver may be responsible for the drastic depletion of FAD levels that precipitates foetal degeneration.

Previously we provided biochemical (Muniyappa & Adiga, 1980*a*) and immunological (Muniyappa & Adiga, 1980*b*) evidence in the pregnant rat for a specific riboflavin carrier protein obligatorily involved in transplacental flavin transport to ensure uninterrupted vitamin supply to the developing foetuses. The functional importance of this maternal vitamin carrier was shown by passive immunoneutralization, which led to drastic curtailment of vitamin influx into the foetoplacental unit, culminating in foetal wastage and hence pregnancy termination (Murty & Adiga, 1981; Krishnamurthy *et al.*, 1984). More recently we demonstrated that one of the major consequences of such acute foetal flavin deficiency induced by immunological interference with the functioning of this maternal protein was a drastic disturbance in relative amounts of embryonic flavin nucleotides, the most conspicuous being the severely depleted FAD content (Krishnamurthy *et al.*, 1984). Whereas the latter aspect of disturbed flavin metabolism is reminiscent of that encountered in a terminally differentiated tissue like the liver of pregnant rats fed either a riboflavin-deficient diet alone or one supplemented with galactoflavin (Miller *et al.*, 1962), or during hypothyroid conditions (Rivlin & Langdon, 1965), the relatively less pronounced changes in foetal FMN and riboflavin concentrations in the face of near-total depletion of FAD suggested that the developing and differentiating foetus may be endowed with a flavin metabolic machinery qualitatively and/or quantitatively different from that of the adult liver. To explore the above possibility, the activities and kinetic properties of the enzymes involved in flavin-nucleotide metabolism of the maternal liver were compared with those of the early foetuses. This also permitted the delineation of the enzymic lesion responsible for deranged flavin-nucleotide metabolism in foetuses consequent on immunoneutralization of the maternal vitamin carrier.

Experimental

Materials

ATP, NADPH, phosphoenolpyruvate, pyruvate kinase, L-lactate dehydrogenase and catalase were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. DL-Alanine was from Koch–Light Laboratories, Colnbrook, Slough, Berks., U.K. The sources of other chemicals and biochemicals were detailed previously (Krishnamurthy *et al.*, 1984). [2-^{14}C]Riboflavin (sp. radioactivity 31 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Adult female albino rats (Wistar, 150 g body wt.) were kept for mating with fertile males and their pregnancies monitored as described previously (Murty & Adiga, 1981). For the assay of catabolic enzymes, namely FMN phosphatase (acid phosphatase, EC 3.1.3.2) and nucleotide pyrophosphatase (EC 3.6.1.9), as well as of biosynthetic enzymes, namely flavokinase (riboflavin kinase, EC 2.7.1.26) and FAD pyrophosphorylase (FMN

adenylyltransferase, EC 2.7.7.2), the animals were killed on day 14 of pregnancy.

In all cases, the rats were killed 24h after the administration of either non-immune rabbit serum (0.5ml/rat) for the control group or the antiserum (0.2ml/rat) to purified riboflavin carrier protein raised in rabbits. This dose of antiserum had previously been shown to be effective for 100% foetal rejection between 24 and 48h of antiserum administration. A similar sequence of events was observed when the antiserum was administered on any day between day 6 and day 16 of pregnancy (Muniyappa & Adiga, 1980*b*). The antiserum to chicken riboflavin carrier protein could neutralize 480μg of protein/ml of serum at equivalence point. Sera were administered intraperitonially to day-13 pregnant rats. At 24h after antiserum administration, drastic foetal wastage, as indicated by 50–60% decrease in fresh weight, was clearly evident. Purification of chicken's-egg riboflavin carrier protein and elicitation of antibodies to the purified protein in rabbits was described previously (Murty & Adiga, 1981).

After the animals had been killed by decapitation, whole foetuses were removed and dissected free of the maternal uterine covering, placenta and the cord. The foetuses were then washed several times with buffer until most of the blood was removed. Before homogenization maternal livers were perfused *in situ* for 10min with the buffer used for the particular enzyme assay until all the blood was removed.

Homogenization was performed at 0–4°C with a Potter–Elvehjem homogenizer with seven to ten strokes of the Teflon pestle. For all enzyme assays, the foetuses and the maternal liver were separately processed at 4°C, unless otherwise stated, and 10–20% (w/v) homogenates were prepared, depending on the enzyme to be assayed.

Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard. Sheep kidney apo-(D-amino-acid oxidase) was partially purified by the method of Burton (1955) and was stored as a freeze-dried powder at 0–5°C until used. One unit of flavokinase or FAD pyrophosphorylase activity is that catalysing the formation of 1nmol of product/h under the experimental conditions employed. Similarly, one unit of nucleotide pyrophosphatase or FMN phosphatase activity is expressed as 1nmol of substrate hydrolysed/min. For all enzymes, the specific and total activities are expressed as units/mg of protein and units/g of tissue respectively. K_m and $V_{max.}$ values were determined by the Lineweaver–Burk plot and the *h* value was calculated from the slope of Hill plot by plotting $\log[(v/V_{max.})-v]$ against $\log s$ on a linear scale (Segal, 1975).

Enzyme assays

FMN phosphatase. The enzyme extract was prepared for assay as described by McCormick & Russel (1962). The assay mixture (1.0ml) contained 7.5mM-potassium acetate buffer, pH5.0, 2mM-FMN and 0.5mg of the enzyme protein (McCormick, 1962). After incubation for 10min at 37°C in subdued light, the reaction was terminated by spotting 100μl of the reaction mixture on to a Whatman no. 3 paper. Flavins were resolved by descending chromatography using butanol/acetic acid/water (12:3:5, by vol.), and riboflavin quantified fluorimetrically with a Perkin–Elmer 203 spectrofluorimeter.

Nucleotide pyrophosphatase. For reproducible quantification, it was found necessary to purify this enzyme activity partially before assay by the method of Krishnan & Rao (1972). The assay mixture (1.0ml) contained 20mM-sodium carbonate/sodium bicarbonate buffer, pH9.7, 0.2mM-FAD and the enzyme protein (0.28mg). The reaction mixture was incubated at 30°C for 1.5min and the reaction terminated by addition of 2 ml of ethanol. The amount of FAD hydrolysed was determined by measuring increase in flavin fluorescence (Bessey *et al.*, 1949).

FAD pyrophosphorylase. FAD pyrophosphorylase was partially purified from the maternal liver as well as from the foetuses as described by DeLuca & Kaplan (1958). The reaction mixture (McCormick, 1964) contained 25mM-potassium phosphate buffer, pH7.5, 1mM-ATP, 1 mM-$MgCl_2$, 0.5mM-FMN and the enzyme preparation (1.0mg) in a total volume of 1.0ml. Incubation was carried out in the dark for 60min at 37°C and the reaction terminated by heating at 80°C for 5min. After removing the precipitated protein by centrifugation, the supernatant was assayed for FAD by the spectrophotometric method of McCormick (1964) as modified by Rivlin (1969*a*), which employed apo-(D-amino-acid oxidase).

Flavokinase. Flavokinase was partially purified by the method of McCormick (1962) and the activity determined as described by Merrill & McCormick (1980) with slight modifications. Assay mixtures (0.5ml) contained 0.1M-Tris/HCl, pH8.2 at 37°C, 1mM-ATP, 1mM-$ZnCl_2$, 0.1mM-[2-^{14}C]riboflavin and the protein (hepatic, 1mg; foetal, 12 mg). After incubation for 60min in the dark at 37°C, portions of the reaction mixture were applied to Whatman no. 3 paper and chromatographed with butanol/acetic acid/water (12:3:5, by vol.). Radioactivities associated with FMN and riboflavin regions were quantified by liquid-scintillation spectrometry (Merrill & McCormick, 1980).

Foetal flavokinase was assayed essentially as

described above, except that the reaction mixture additionally contained an ATP-regenerating system (20 μg of pyruvate kinase and 1 mM-phosphoenolpyruvate).

Under the conditions of the assay employed, all the reaction rates were linear with time and proportional to the amount of enzyme protein used. The substrate concentrations employed gave optimal activities.

Results

FMN phosphatase activities in the maternal liver and the foetus

The kinetic properties of the enzyme from the two tissues are summarized in Table 1. Hepatic as well as foetal FMN phosphatase showed comparable h values. Further, both the specific and total activities were unaltered by passive immunoneutralization of maternal riboflavin carrier protein (Table 2). The K_m values of the enzyme for FMN differed only marginally, being 0.3 mM and 0.7 mM for liver and foetus respectively.

Nucleotide pyrophosphatase activities in the two tissues

As shown in Table 1, the K_m for FAD is almost the same for the foetus enzyme as for that from maternal liver. However, the foetal pyrophosphatase activity is about 4-fold higher than its hepatic counterpart in terms of both specific as well as total activity. Interestingly, no significant alteration in the activities of the enzyme was noticeable on immunoneutralization of riboflavin carrier protein in the mother (Table 2).

Flavokinase activities in the foetal and the hepatic tissues

The K_m of the hepatic flavokinase for riboflavin was 14 mM as compared with 70 μM for the foetal enzyme (Table 1). It may be mentioned that the foetal flavokinase could be assayed only after the inclusion of an ATP-regenerating system in the reaction mixture, which is suggestive of intrinsically higher ATPase activity, thus necessitating a continuous supply of ATP for FMN production in the foetal tissue. It is also noteworthy that, in control animals, the hepatic flavokinase activity was severalfold higher than its foetal counterpart; however, under conditions of immunoneutralization there was a 5–6-fold increase in foetal flavokinase activity over the controls, whereas hepatic activity remained unaltered under these conditions (Table 2).

FAD pyrophosphorylase in the two tissues

The K_m values for FAD of the foetal and the hepatic enzymes were found to be 0.3 and 0.01 mM-

Table 1. *Kinetic properties of the foetal and maternal hepatic enzymes of flavin-nucleotide metabolism*

Results are means ± S.E.M. ($n = 5$). K_m, $V_{max.}$ and h values were determined as described in the text.

Enzyme	Nucleotide pyrophosphatase		FMN phosphatase		Flavokinase		FAD pyrophosphorylase	
Kinetic property / Source	Foetus	Maternal liver	Foetus	Maternal liver	Foetus	Maternal liver	Foetus	Maternal liver
K_m for substrate (mM)	0.4 ± 0.03	0.3 ± 0.02	0.7 ± 0.04	0.3 ± 0.02	0.07 ± 0.01	0.01 ± 0.002	0.3 ± 0.02	0.01 ± 0.005
Hill coefficient (h)	1.0	0.9	0.9	1.0	0.9	0.8	1.0	0.8
$V_{max.}$	95 ± 3.0*	8 ± 1.0*	42 ± 1.8*	33 ± 2.0*	0.25 ± 0.04†	2.85 ± 0.4†	0.1 ± 0.01†	0.24 ± 0.02†
$V_{max.}/K_m$	237	31	64	116	4	203	0.3	24.0

* nmol·min^{-1}·mg of protein^{-1}.
† nmol·h^{-1}·mg of protein^{-1}.

Table 2. *Influence of administration of rabbit antiserum to chicken's-egg riboflavin carrier protein on hepatic and foetal flavin-nucleotide-metabolizing enzymes in the pregnant rat*

Results are means ± S.E.M. ($n = 5$). The increase in foetal flavokinase and the decrease in foetal FAD pyrophosphorylase activities in the antiserum-treated animals was statistically significant ($P < 0.01$ as compared with controls by Student's t test). Details of the enzyme assays are given in the text. The protein contents of the foetus and the maternal liver were 100 mg and 140 mg/g of tissue respectively.

Parameter	Enzyme ... Nucleotide pyrophosphatase		FMN phosphatase		Flavokinase		FAD pyrophosphorylase	
	Source ... Foetal	Hepatic	Foetal	Hepatic	Foetal	Hepatic	Foetal	Hepatic
Specific activity*								
Control	46.0 ± 9.2	13.2 ± 4.0	18.6 ± 1.6	11.0 ± 0.6	0.12 ± 0.01	4.0 ± 0.8	0.11 ± 0.004	0.26 ± 0.01
Antiserum-treated	51.0 ± 4.2	9.2 ± 3.6	18.0 ± 1.0	13.0 ± 1.8	0.6 ± 0.16	4.2 ± 0.6	0.05 ± 0.001	0.25 ± 0.01
Total activity†								
Control	3.0 ± 0.8	0.98 ± 0.4	0.84 ± 0.04	0.67 ± 0.2	0.5 ± 0.1	22.2 ± 0.4	13.0 ± 0.4	29.0 ± 2.0
Antiserum-treated	3.12 ± 0.6	0.74 ± 0.26	0.78 ± 0.09	0.65 ± 0.17	2.0 ± 0.6	22.0 ± 0.8	9.4 ± 0.2	28.6 ± 1.4

* Units/mg of protein.
† Units/g of tissue.

respectively (Table 1). The higher K_m of the foetal pyrophosphorylase for FMN is suggestive of a lower affinity of the enzyme for its substrate. The hepatic pyrophosphorylase level was 2-fold higher than its foetal counterpart and was unchanged on immunoneutralization of riboflavin carrier protein in the pregnant rat. Contrastingly, a 50% decrease in the foetal enzyme activity was manifest under the above conditions (Table 2). Since both specific and total activities exhibited parallel trends, it would appear that the decreased enzyme activity in the affected foetuses reflects the situation *in vivo* and is not a consequence of accelerated depletion of its protein content by, presumably, catabolism.

Discussion

One of the remarkable features of the acute flavin deficiency precipitated in the developing foetus as a consequence of immunological interference with the maternal vitamin carrier is that, instead of entering a quiescent stage due to developmental arrest, the foetus undergoes rapid degeneration, culminating in death. This contrasts with the situation in certain tumours where vitamin deficiency inhibits growth rather than bringing about degeneration; the tumour tissue is apparently more resistant than normal organs to deficiency (Rivlin, 1973). Similarly, in adult animals, dietary vitamin deficiency only suppresses growth rate without causing immediate death. These observations seem to suggest subtle differences in detail between the enzymes concerned with flavin metabolism and regulation thereof in the adult liver and foetus. The present investigations largely support the above premise. Thus, with regard to flavin-nucleotide-biosynthetic enzymes, the significantly higher K_m of the foetal FAD pyrophosphorylase for FMN *vis-à-vis* its hepatic counterpart (Table 1) is suggestive of a need for a relatively higher intracellular FMN concentration to drive the reaction in favour of FAD synthesis. From the catalytic indices (represented by the $V_{max.}/K_m$ ratio), it is clear that both the biosynthetic enzymes of flavin-nucleotide metabolism from the maternal liver are relatively more efficient than their foetal counterparts (Table 1). Thus it would appear, perhaps surprisingly, that, at the early stages of foetal growth, these anabolic enzymes are relatively sluggish in activity and less adapted to their function. A plausible explanation for this finding may be concerned with the fact that these biosynthetic enzymes in the adult tissue are modulated by the thyroid hormones (Greengard, 1971); on the other hand, in developing rodent foetuses the thyroid becomes gradually functional only during days 16–20 of gestation. It was also shown that these enzymes increase slowly

during foetal development to reach adult levels just after birth (Rivlin, 1969*b*).

In contrast with the anabolic enzymes, the K_m values of the foetal flavin-degrading enzymes, namely nucleotide pyrophosphatase and FMN phosphatase, are more-or-less comparable with their hepatic counterparts (Table 1). Furthermore, whereas FMN phosphatases of the two tissues are similar in terms of their catalytic indices, the foetal nucleotide pyrophosphatase has a 7–8-fold higher catalytic efficiency than the corresponding maternal liver enzyme. It would appear therefore that, superimposed on an inherently inefficient biosynthetic machinery concerned with the elaboration of flavin coenzymes, acute flavin deficiency precipitated by immunoneutralization of the carrier protein brings about a 50% decline in foetal FAD pyrophosphorylase in the treated mothers (Table 2). It is possible that a corresponding increase in foetal flavokinase (Table 2) may represent an adaptive, but unsuccessful, foetal stratagem to offset the depressed FAD levels. It is noteworthy that, unlike the situation in the flavin-deficient adult liver, the foetal FAD is not conserved at the expense of the more dispensable FMN by an increase in FAD pyrophosphorylase (Rivlin, 1969*a*). It would also appear that the FAD pyrophosphorylase of the foetus is the pace-setting enzyme in flavin-nucleotide biosynthesis and is more stringently regulated by the available intracellular riboflavin. It is intriguing that the degradation of FMN and FAD in foetuses seems to be independent of riboflavin status, since the degrading enzymes remain unaltered both in flavin-deficient foetuses and in the vitamin-deficient-adult hepatic tissue (Lee & McCormick, 1983; Fass & Rivlin, 1969).

From the abovementioned observations, it is clear that there are certain basic differences in enzyme profiles concerned with flavin-nucleotide metabolism between the early foetus *vis-à-vis* the adult liver; in particular, the foetal biosynthetic enzymes seem to be relatively sluggish, whereas the reverse seems to be the case with the catabolic enzymes. Although it is conceivable that some of the differences in enzyme characteristics observed are related to the different nature of the tissues used for comparison, i.e. liver in the mother and whole-body organs in the foetus. Interference with the carrier-protein-mediated transplacental vitamin-delivery mechanism, with the resultant acute flavin deficiency (Murty & Adiga, 1981; Krishnamurthy *et al.*, 1984), seems to inhibit FAD pyrophosphorylase selectively, leading to curtailed FAD production. This situation, coupled with relatively higher activities of FAD-catabolizing enzymes in the foetus, apparently depletes the vital coenzymes to such critically low levels that the affected foetus is compelled to degenerate rapidly in the absence of other regulatory (hormonal?) mechanisms to offset this derangement. Further experiments on the dynamics of flavin flow into different flavin coenzymes in the affected foetuses should substantiate the above conclusion.

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