L-GULONATE DEHYDROGENASE : A COMMON REGULATORY TARGET IN DIABETIC NEPHROPATHY, RETINOPATHY, AND IN OVARIAN FUNCTION

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Nutritional studies on humans and animals have long back established the role of vitamins and mineral elements in health[1]. For many phylogenic groups these are essential i.e. they have to be supplied in the diet as, such organisms like humans have lost the ability to biosynthesize these dietary components. While a lot of biochemical work has been done on the dietary carbohydrate, protein and lipid metabolism in generating, storing and utilizing energy for living processes[2], the metabolic link between the biochemical reaction in which the vitamin takes part and the disease precipitated in their absence is not clearly understood. This is true for many genetic disorders also where establishing cause and effect metabolic link between gene defect and a phenotype like mental disorder is proving to be difficult. Reproduction in higher animals is a complex physiological process resulting in progeny generation[3]. Vitamins like vitamin E (alpha tocopherol) are essential for successful reproduction in many higher animals[4]. Other vitamins like ascorbic acid, riboflavin, niacin and thiamine directly or indirectly are required for reproductive success. In oviparous animals, these vitamins are supplied by the female adult through the egg yolk to the new generation during development[5]. But during the adult stage, they have to be obtained through diet. Reproductive hormones of pituitary and gonadal origin regulate many reproductive physiological processes[6,7]. One of the known actions of the pituitary luteinizing hormone (LH) is to cause depletion of the ovarian ascorbic acid content and this in effect forms the basis of a reliable bioassay for this hormone[7]. What is not known clearly is the mechanism by which this depletion is brought about. In animals like rats and mice, it could be envisaged that LH must be affecting, either the biosynthesis or catabolism or both, of ascorbic acid in the ovary. The famous 'Parlow rat' is the experimental model to demonstrate this bioassay. Briefly 25-26 day old immature rats of Holtzman or any other strain are administered Pregnant Mare Serum Gonadotrophin (PMSG) and human Chorionic Gonadotrophin (hCG) in sequence to accelerate ovarian follicular growth (hyperplasia and hypertrophy) and induce super ovulation (Fig 1).

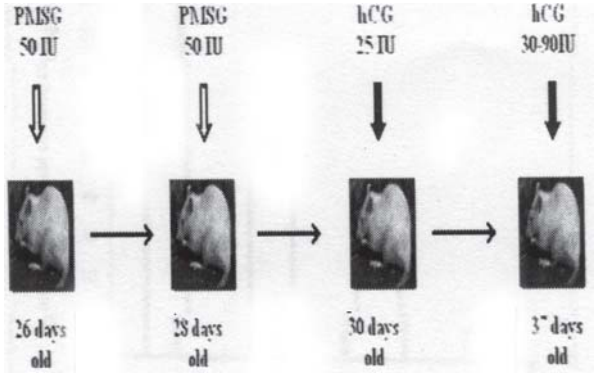

Fig. 1. Ovarian ascorbic acid depletion assay as proposed by A.F. Parlow (1961).

Five to seven days after the administration of hCG, the animals receive the standard doses of LH or unknown substance suspected of possessing LH like activity. Four hours later the animals are sacrificed and the ovarian ascorbic content is estimated (Fig 2).

Lecture delivered on July 9th, 2009, on the occasion of ISNA at Kolkata

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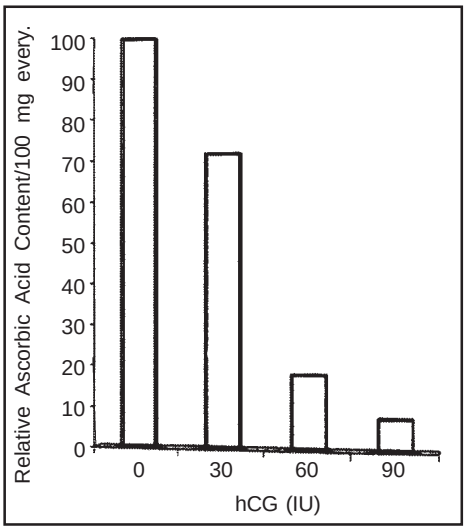


Fig. 2. Dose response to hCG in OAAD assay.

As said earlier the signalling pathway for this effect of LH is not understood. Our preliminary experimental results indicated that cAMP (Adenosine-3', 5' cyclic monophosphate), the famous second messenger could mediate this action of LH[8] (Table 1). It was also shown to be cycloheximide insensitive (Table 2).

TABLE 1. Effect of cyclic nucleotides on ovarian ascorbic acid content.

Group No.	Treatment*	Dose (μg/rat)*	Ascorbic acid Mg (%) (Mean ± S.D.)	Change in Ascorbic acid (%)
I	Saline	–	77.11 ± 9.5	0.00
II	5'-AMP	20	79.69 ± 5.1	+3.35[a]
III	dbcAMP	5	71.87 ± 2.6	-6.79[b]
IV	dbcAMP	20	73.40 ± 5.1	-4.81[c]
V	eGMP	20	61.65 ± 5.1	-20.04[d]
VI	saline	–	89.84 ± 11.3	0.00
VII	dbcAMP	50	77.84 ± 8.5	-13.36[e]

* Wistar strain rats were used.

a p > 0.1; d.f. = 10 (I:II)

b p > 0.1; d.f. = 8 (I:III)

c p > 0.1; d.f. = 9 (I.IV)

d p < 0.025; d.f. = 9 (I:V)

e p = 0.05; d.f. = 10 (VI:VII)

Ascorbic acid prevents apoptosis in mice follicles exposed to folicle stimulating hormone (FSH) in serum-free media. It is reported to promote follicular integrity. It does not affect estrogen production or follicular growth.

TABLE 2. Inability of cycloheximide to block LH action on ovarian ascorbic acid.

Group No.	Treatment*	Dose (μg/rat)*	Ascorbic acid Mg (%) (Mean ± S.D.)	Depletion (%)
I	Saline	–	89.84 ± 11.20	0.00
II 43.68[a]	LH	3.2	50.59 ± 5.02	
III 40.67[b]	LH+Cyclo-heximide	3.2 + 250	53.36 ± 11.83	

* Wistar strain rats were used.

a. p < 0.005; d.f. = 9 (I:II)

b. 0.25 > p> 0.1; d.f. = 9 (II:III)

It may have a role in remodelling of follicular cells. Metalloproteinase 1 inhibitor and metalloproteinase 2 tend to increase in ovarian follicles by the addition of ascorbic acid. Ascorbic acid does not appear to have progesterone like function. It is known to prevent apoptosis in ovary. LH induced luteolysis results in increase in lipid peroxidation, decrease in ascorbic acid and decrease in

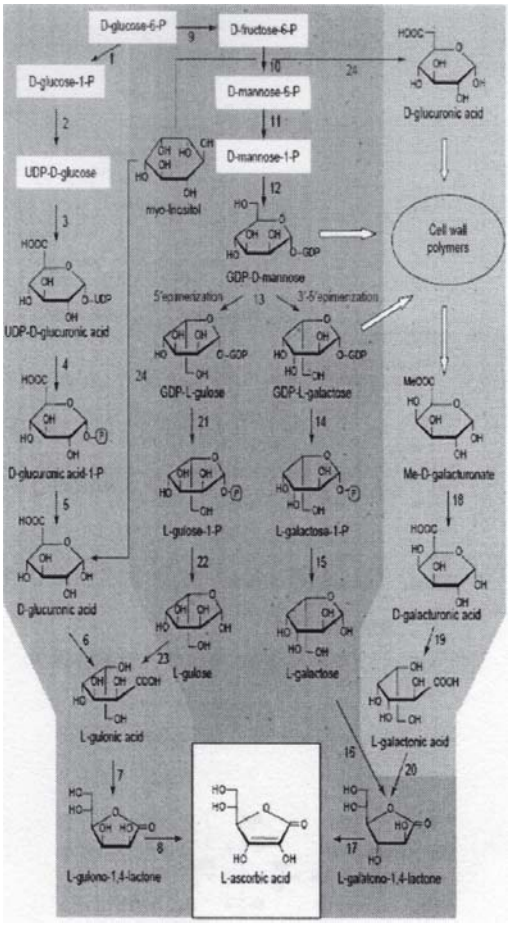


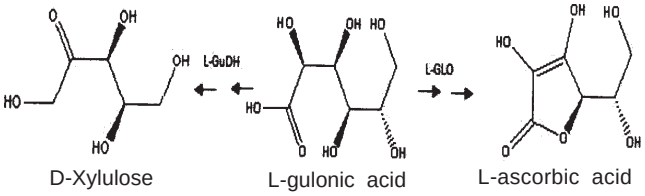
Figure 3. Different biosynthetic pathways of L-ascorbic acid in animals (reactions 1–8) and plants (reactions 9-24). Adapted from, V. Valpuesta and M.A. Botella, Trends Plants Sci., 9,573 (2004).

progesterone. LH increases estrogen but decreases ascorbic acid content in ovaries[8]. Ascorbic acid augments ovulation induction by clomiphene.

A perusal of ascorbic acid metabolism in animals[10], plants[11] and microbes indicate that more than one metabolic pathway exists for the biosynthesis of ascorbic acid in different phylogenic groups (Fig 3).

A common feature is that the immediate precursor metabolite in the biosynthetic pathway is L-gulonolactone which gets converted to L-ascorbic acid via 2-keto L-gulonolactone and this reaction is catalysed by L-gulonolactone oxidase (LGO)[12]. This enzyme is apparently absent in humans, other primates, guineapigs and fist1[2]. It is also known that ascorbic acid undergoes an interesting catabolic transformation to oxalate and L-Threose via dehydroascorbate and diketoascorbate[13]. The enzymatic details are not clear. Ascorbic acid oxidase, however, inactivates ascorbate by oxidising it to dehydroascorbate. While outside the living tissue, ascorbate easily gets oxidised by air, there are reports that inside tissues, the equilibrium lies towards ascorbate[11]. However this is not substantiated well. L-gulonolactone is derived from L-gulonic acid or D-glucuronolactone or even D-galacturono lactone[12]. In the case of conversion of D-glucuronic acid to L-gulonic acid there is inversion as these are not mirror images. But in the case of conversion of D-galacturonic acid to L-galactonic acid, there is no inversion[13]. It is evident that other important enzymes in this pathway from D-glucuronic acid are D-glucuronate reductase, L-gulonate dehydrogenase and the lactonase. These facts raise many questions about the possible effects of LH on the enzymes of ascorbic acid biosynthesis and degradation in the rat ovary. One notices also that the uronate pathway of glucose degradation via myo inositol, D-glucuronic acid and L-gulonate normally leads to the formation of L-xylulose and then to xylitol (Fig 4).

This places L-gulonic acid at a crucial metabolic traffic junction where it can get metabolised to either xylitol or ascorbic acid.



The question that arises is what external factors control this channelling of L-gulonate into either of the two possible pathways ? Alternately, one can envisage a situation where L-gulonate is not transformed to ascorbate under normal conditions. Under special conditions, it is possible that L-GuDH is blocked, thus formation of xylitol is blocked and hence ascorbate content is increased. Does such a situation in reverse occur in the ovary ? LH causes depletion of ascorbic acid in the ovary. Hence either the biosynthesis is blocked or the degradation is accelerated by the putative external factor. What is the effect of LH on rat ovarian ascorbic acid oxidase, L-gulonolactone oxidase, L-GuDH, and D-glucuronate reductase etc. ? The more interesting fact is that excessive formation of xylitol and excretion through kidney is one of the factors leading to precipitation of diabetic nephropathy in chronic diabetes[14]. In order to understand this problem, hog kidney L-GuDH was purified to homogeneity. Data related to part of the purification process is given in Table 3.

Our experience showed that approximately one unit of this enzyme activity was present in one gram of the tissue (Table 4).

TABLE 3. Hydroxyapatite column enables purification of L-Gulonate Dehydrogenase from Kidney.

S.No.	Sample	Volume (mL)	Protein (mg)	Activity (µmol/min)	Specific Activity (U/mg)	Yield* (%)	Purification Fold
1.	Cytosol	580	6845	11.88	0.0017	100	1.00
2.	$(NH_4)_2SO_4$ ppt (60% S)	138	3844	150.22	0.0391	1264	22.51
3.	HA unbound	200	1294	211.45	0.1635	1779	94.18

*Yield increase upon salt fractionation indicates possible presence of inhibitor in the cytosol. The nature of the inhibitor is yet to be investigated

TABLE 4. Comparison of tissue and recombinant enzyme.

Initial activity*	Yield (%)	Specific activity of the final preparation (U/mg protein)
t-enzyme	*	1 Unit/gram tissue/34 mg protein
r-enzyme	48	55 Units/Litre/78 mg protein

*Every of t-enzyme strangely goes more than 100% due to increase after a certain fractionation.

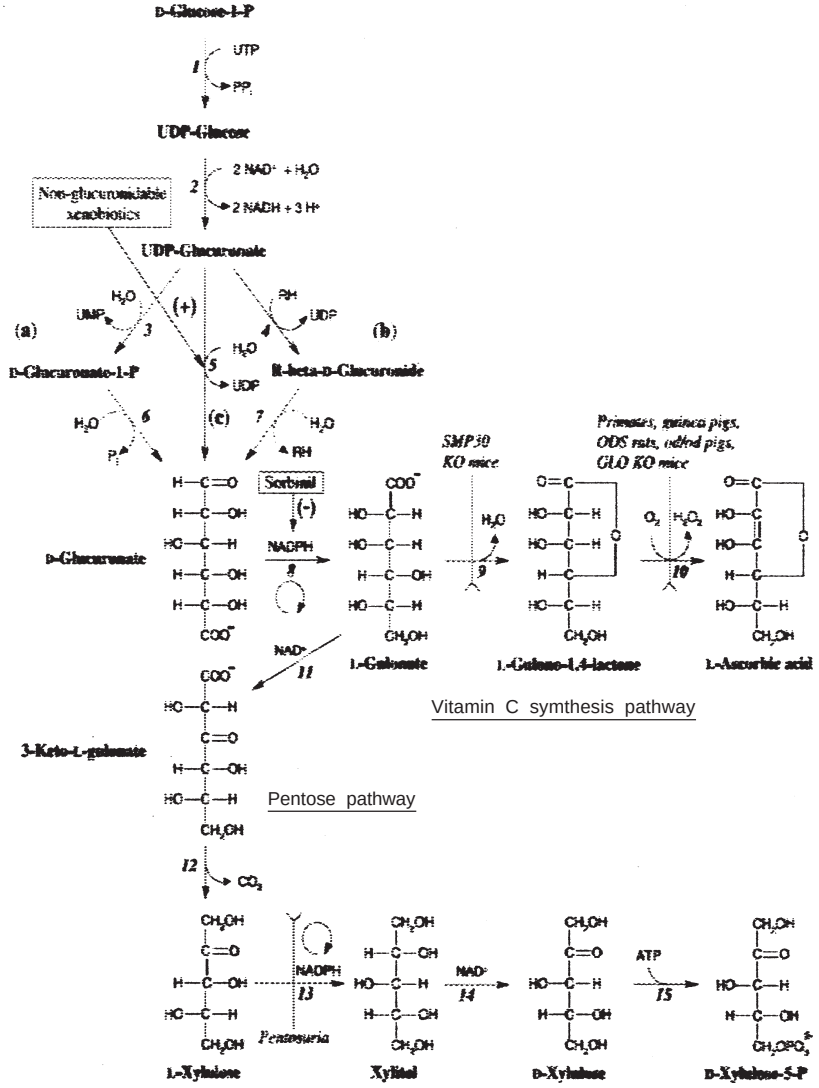


Figure 4. Vitamin C biosynthesis pathway and pentose pathway in animals. Adapted from C.L. Linster and E.V. Schaftingen, *FEBS J.*, 274, 1 (2007).

TABLE 5. Purification of Recombinant L-GuDH with His-tag.

S.No.	Sample	Volume (mL)	Protein (mg)	Activity (μmol/min)	Specific Activity (U/mg)	Yield* (%)	Purification Fold
1.	Bacterial Extract	40	156	0.111	0.7	100	1.00
2.	$(NH_4)_2SO_4$ ppt (60% S)	16	75	0.057	0.8	51	1.07
3.	Blue-agarose Unbound	32	59	0.062	1.0	56	1.18
4.	HA unbound	125	19	0.06	3.2	54	4.46
5.	HA unbound concentrate	1.1	16	0.055	3.4	49	4.89
6.	S-200 pool I	3.5	6.5	0.06	9.44	48	13.4

*Later it was found that in the case of the r-enzyme without His-tag, one can skip the HA step and go directly to DE-52 and S-200 to get the pure enzyme.

We could get more than 245 fold purification by including additional steps like DE-52 and S-300 chromatographies. The final preparation had a specific activity of 7 units per mg protein. The cDNA for L-GuDH was also made and cloned without and with HIS-tag in pET 17b and pET 28a vectors (data not shown). The recombinant enzyme, purified 13.4-fold from crude bacterial extract (Table 5).

It had a specific activity of 9 units per mg protein. An interesting property of the hog kidney L-GUDH must be noted. Although reports exist in literature that rabbit eye L-GUDH can be purified on Blue-sepharose, it was observed that the hog kidney enzyme, both native and the recombinant types, did not bind to Blue-4 or Blue-2 sepharose. This is puzzling as the enzyme is a pyridine-linked dehydrogenase (Table 6).

The native and the recombinant enzymes were characterised with regard to physico-chemical and immunological properties (data not shown). The enzyme could oxidise, in additon to L-gulonate (The K_M Value was between 2.10 mM to 2.75 mM), D-gluconic acid, L-malic acid and beta hydroxyl butyric acid (Table 7).

The reported activity of this native enzyme on L-arabonic acid is not easily explainable[14]. There were other interesting aspects of its substrate specificity. For example the recombinant L-GUDH had significant activity on L-malic acid and L-threonone but the native enzyme did not act on these substrates (Table 7).

It was also observed that four naturally occurring

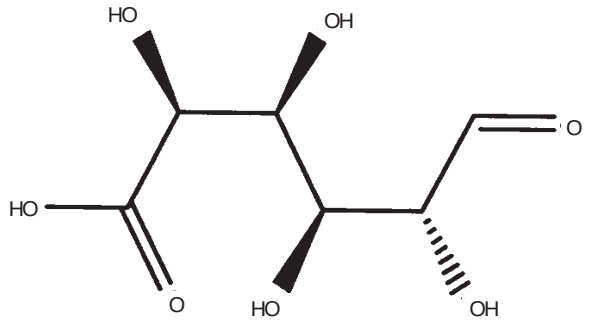
TABLE 6. Recombinant L-GuDH does not bind Cibacron Blue

Sample	Affinity Column	Unbound/Bound Activity (%)
ALR-1	Blue-4-agarose	70/33
r-L-GuDH	Blue-4-agarose	>95/0.0
r-L-GuDH	Blue-4-agarose (5mM pH 8.0)	>95/0.0
r-L-GuDH	Blue-2-agarose	>95/0.0
BSA	Blue-2-agarose	0.0/100

Table 7. Activity against different substrates

Substarte	Activity (%)	
	(t-enzyme)	(r-enzyme)
L-Gulonic acid	100	100
L-Malic acid	0	27
D-Gluconic acid	36	N.D.
L-Thereonine	0	10-15
R(-) 3-OH butyric acid	6	N.D.

metabolites inhibited the enzyme to varying extent i.e. 55% to 100% (data not shown). However, in one case, i.e. D-Glucoronic Acid (Fig. 5), the K_i was 12.9 mM and the inhibition was of a mixed type.



D-glucuronic acid

Fig 5. D-Glucoronic Acid, a powerful inhibitor of L-GuDH.

Metabolism of phosphorylated sugars has drawn more attention from scientists that of non-phosphorylated sugars. Many eye lens crystallins have been shown to possess gulonate dehydrogenase activity[15]. When the crystallin gene from rabbit eye lens was cloned and the cDNA sequenced, it matched with that of kidney gulonate dehydrogenase[15].

Xylose feeding to adult rats has been shown to result in cataract[16]. But aldose reductase activity in the adult rat eye lens is not significant to account for xylitol formation which is necessary for the manifestation of cataract condition. In young rats the same xylose feeding does not precipitate any cataract formation. It was suggested, using ^{14}C-labelled sugars, that the *myo* inositol metabolism to xylulose, the equilibrium and conversion of fed xylose to xylulose and the action of L-iditol-NAD^+ oxidoreductase causes the formation of xylitol and thus cataract[16].

In conclusion, studies from many laboratories including ours have shown that L-gulonate dehydrogenase is an important enzyme which can control the metabolite flux towards either of the metabolic pathways (formation of ascorbic acid or xylitol) at a crucial metabolic traffic junction. ❐

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