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11. The three polyvalent ascitic fluids from immunized mice were furnished by the Research Resources Branch of National Institute of Allergy and Infectious Diseases, National Institute of Health. The polyvalent Palyam ascitic fluid was prepared from the following viruses: Palyam (strain IG 5287); I 68886 (strain I 68886); IG 15534 (strain IG 15534); Corriparta (strain MRM 1); Eth Ar 1846-64 (strain Eth Ar 1846-64); Eubenangee (strain IN 1074); Dak Ar B 1327 (strain Dak Ar B 1327); D'Aguilar (strain Aus B 8112). The polyvalent group Kemerovo ascitic fluid was prepared from the following viruses: Kemerovo (strain Rio); Chenuda (strain EgAr 1152); Mono Lake (strain Cal Ar 861); Wad Medani (strain EgAr 492); Tribec (prototype strain); Huacho (strain Cal Ar 883). Polyvalent 8 ascitic fluid was prepared from the following viruses: blue tongue (strain BT8); EHD (New Jersey original strain); Ib Ar 22619 (strain Ib Ar 22619); Changuinola (strain BT 436); Irituia (strain Be An 28873); Colorado tick fever (Condon strain).
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Type 2 Hyperprolinemia: Absence of Δ^1-Pyrroline-5-Carboxylic Acid Dehydrogenase Activity

Abstract. *Δ^1-Pyrroline-5-carboxylic acid dehydrogenase activity was measured radioisotopically in normal and type 2 hyperprolinemia fibroblasts. The type 2 cells had no detectable activity over a range of reaction conditions whereas normal cells had easily measurable activity. This enzymatic defect accounts for the biochemical abnormalities in type 2 hyperprolinemia.*

The degradation of proline in man involves the conversion of proline to Δ^1-pyrroline-5-carboxylic acid (PC) by proline oxidase and the oxidation of PC to glutamic acid by PC dehydrogenase (*1*) (Fig. 1). Individuals with abnormalities in this pathway have been classified into two groups, on the basis of plasma and urinary findings, as type 1 and type 2 hyperprolinemia (*2*). Both types are characterized by elevated concentrations of proline in the plasma with resultant iminoglycinuria. Although plasma proline concentrations are of greater magnitude in type 2 hyperprolinemia, the distinguishing feature between the two types is that individuals with type 2 excrete large amounts of an *o*-aminobenzaldehyde reactive material, presumably PC, in their urines.

The metabolic defect in type 2 hyperprolinemia has been shown, in a single patient, to be a deficiency of proline oxidase (*3*). The enzymatic defect in type 2 hyperprolinemia, however, has not been defined. We now report that type 2 hyperprolinemia is due to an absence of PC dehydrogenase activity.

The patient is a 13-year-old asymptomatic Mexican-American female. She exhibits the characteristic biochemical abnormalities of type 2 hyperprolinemia, namely, a plasma proline concentration of 1.658 to 2.534 μmole/ml (normal, 0.07 to 0.15 μmole/ml), marked iminoglycinuria, and the presence of *o*-aminobenzaldehyde reactive material in her urine (*4*).

Using standard techniques, we obtained skin fibroblasts from the patient (HP type 2). Control cells included skin fibroblasts obtained from a 2-year-old white male with G_{M1} gangliosidosis (CJ) and normal fibroblasts from a human male (D550) and a human female (D551) from the American Type Culture Collection. All cells were grown in Eagle's minimal essential media with added nonessential amino acids, neomycin, glutamine, and 10 percent fetal calf serum. The concentration of proline in the medium was 0.1 m*M*. Cultures were routinely checked for bacterial and mycoplasmal contamination and were negative throughout the period of investigation.

To prepare enzyme extracts, we harvested the cells in the late log phase of growth; the cells were then washed and sonicated in a small volume of 50 m*M* tris buffer, *p*H 8.2, with 1 m*M* EDTA. The PC dehydrogenase activity of the extracts was determined by measuring the conversion of ^{14}C-labeled PC to ^{14}C-labeled glutamate (*5*). The reaction was stopped at 30 minutes by the addition of HCl, the final concentration being 1*N*. This acidified mixture was then mixed with an equal volume of a solution of *o*-aminobenzaldehyde (10 mg/ml in 10 percent ethanol and 1*N* HCl). The *o*-aminobenzaldehyde quantitatively combines with unreacted PC, forming a dihydroquinazolium compound which remains at the top of a Dowex column (150 by 5 mm), while the reaction product, ^{14}C-labeled glutamate, is eluted with 1*N* HCl. At reaction conditions of *p*H 8.2 and 30°C, this assay is linear up to 75 μg of added fibroblast protein and up to 30 minutes (for the reaction time). The recovery of glutamate is greater than 90 percent.

Ornithine δ-transaminase and PC reductase were assayed as described (*6*). Protein was determined by the method of Lowry *et al.* (*7*).

Table 1 shows the activity of PC dehydrogenase in control and type 2 hyperprolinemia fibroblasts. The mean activity of the control was 37.0 $\pm$ 3.6 nmole of glutamate produced per hour per milligram of protein, a value that represents about a tenfold increase in radioactivity as compared to either nicotinamide adenine dinucleotide (NAD) or enzyme blanks. In contrast, we were unable to detect any activity in HP type 2 extracts even with the addition of up to 180 μg of extract protein. Furthermore, we altered the reaction mixture by increasing PC concentrations 100-fold or by substituting nicotinamide adenine dinucleotide phosphate (NADP) for NAD, but we were still unable to detect PC dehydrogenase activity in the type 2 extracts. Thus over a range of reaction conditions, extracts of the type 2 hyperprolinemia cells have no detectable PC dehydrogenase activity.

The possibility of an inhibitor of PC

dehydrogenase activity in the type 2 extracts was eliminated by experiments showing that the activity of a small amount of D551 extract was unchanged with the addition of sixfold more type 2 extract (Table 1). We also considered the unlikely possibility that some glutamate-utilizing enzyme present in the type 2 extracts was consuming ^{14}C-labeled glutamate formed during enzyme incubation. We examined this possibility by adding ^{14}C-labeled glutamate and reduced NAD (NADH) (0.01 m*M*) to standard reaction mixtures containing either HP type 2 or D550 extract; we found that 90 percent of the glutamate added at the beginning of the reaction was still present as glutamate at the end of the reaction. This result ruled out excessive consumption of product glutamate in the type 2 extract.

To ensure that the absence of dehydrogenase activity was a specific defect in the HP type 2 cells, we assayed two proline biosynthetic enzymes, ornithine δ-transaminase (OTA) and PC reductase in the HP type 2 and D551 extracts. The activities of OTA (in nanomoles per hour per milligram of protein ± 1 standard deviation) were 164 ± 5 and 159 ± 4 for the type 2 and D551 extracts, respectively. The PC reductase activities (nanomoles per hour per milligram of protein ± 1 S.D.) were 544 ± 5 and 538 ± 27 for HP type 2 and D551, respectively. These studies showed that the absence of PC dehydrogenase in HP type 2 occurred when normal OTA and PC reductase activities were present (*8*).

Thus our findings indicate that type 2 fibroblasts lack PC dehydrogenase. All the biochemical abnormalities in type 2 hyperprolinemia can be explained by the resultant metabolic block. A portion of the PC accumulated because of this block was excreted in the urine and gave rise to the positive urine *o*-aminobenzaldehyde reaction. Most of the accumulated PC, however, is converted to proline. The ratio of proline to PC probably reflects the relative K_m's (Michaelis constant) and V_{max}'s (velocity maximum) of PC reductase and proline oxidase. Our data (*9*) and those of Peisach and Strecker (*10*) show that PC reductase is present in liver in greater amounts and has a lower K_m than does proline oxidase (K_m's are $2 \times 10^{-4}M$ and $2 \times 10^{-3}M$, respectively). Thus if there are adequate amounts of NADH, it is likely that the major fraction of accumulated PC will be converted to proline.

Table 1. Δ^1-Pyrroline-5-carboxylic acid dehydrogenase activity in fibroblast extracts. All determinations were performed in triplicate. Values for each cell line represent the means ± 1 S.D. of determinations performed on at least two extracts.

Extract	PC dehydrogenase (nanomoles per hour per milligram of protein)
CJ	32.9 ± 0.8
D550	39.6 ± 0.5
D551	38.6 ± 2.4
Mean of controls	37.0 ± 3.6
HP type 2	None detectable
HP type 2 plus D551*	37.9

* The assay mixture included 16 μg of D551 extract and 93 μg of HP type 2 extract.

Biochemical examination of the urine of our type 2 hyperprolinemia patient has shown the presence of two *o*-aminobenzaldehyde reactive substances: PC and Δ^1-3-hydroxypyrroline-5-carboxylic acid (3-OHPC), the oxidized breakdown product of hydroxyproline (*4*). In bovine liver PC dehydrogenase utilizes either of these compounds as substrate (*1*, *10*). The finding of 3-OHPC in the urine of our patient provides in vivo evidence that both PC and 3-OHPC are oxidized by PC dehydrogenase.

In view of the proline oxidase defect in type 1 hyperprolinemia and our finding of a PC dehydrogenase defect in type 2 hyperprolinemia, it is interesting that in type 2 the plasma proline concentrations are much higher than in type 1 (*2*). A partial rather than complete deficiency of proline oxidase in type 1 may account for this difference. However, studies on a single case of type 1 showed that the defect was virtually complete (*3*). Alternatively, PC could have a positive regulatory influence on proline biosynthesis. A stimulatory influence by an intermediate in a biosynthetic pathway, however, is uncommon. An interesting third possibility is that a significant amount of PC formed from ornithine by OTA is converted directly to glutamate by PC dehydrogenase. In effect this pathway would provide a bridge between the urea cycle and the Krebs cycle and allow significant amounts of PC to bypass proline and be converted directly to glutamate. In type 2 hyperprolinemia the PC dehydrogenase defect would produce a block in this pathway as well as in proline degradation and result in greater accumulation of PC which, in turn, is converted to proline.

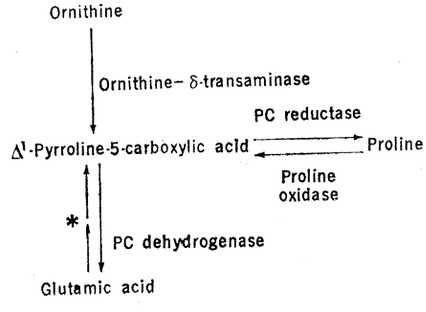

Fig. 1. Biosynthetic and degradative enzymes of proline metabolism. The asterisk indicates enzyme or enzymes not defined in higher organisms.

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