Enzymes Metabolizing Δ^1 -Pyrroline-5-carboxylate in Rat Tissues

By ANNEMARIE HERZFELD,* VASEK A. MEZL† and W. EUGENE KNOX

*Department of Biological Chemistry, Harvard Medical School, and the
Cancer Research Institute of the New England Deaconess Hospital, Boston, MA 02215, U.S.A.*

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The direction and capacity for the metabolism of Δ^1 -pyrroline-5-carboxylate in a number of rat tissues were investigated by measuring the activities of Δ^1 -pyrroline-5-carboxylate reductase, Δ^1 -pyrroline-5-carboxylate dehydrogenase and proline oxidase. Each of these enzymes catalysed unidirectional reactions in which Δ^1 -pyrroline-5-carboxylate was either the substrate or product. Δ^1 -Pyrroline-5-carboxylate reductase activities that were much higher than any previously reported were obtained by avoiding its inactivation in the cold. Δ^1 -Pyrroline-5-carboxylate dehydrogenase, previously said to act on both D- and L-isomers of Δ^1 -pyrroline-5-carboxylate, acted only on the L-isomer. Proline oxidase could not be measured in two adult tissues, in which an inhibitor appeared after birth. The activity of Δ^1 -pyrroline-5-carboxylate reductase significantly paralleled that of ornithine aminotransferase in 23 tissues, showing a widespread potential for proline synthesis from ornithine. An independently distributed potential in fewer tissues for proline degradation to α -oxoglutarate was shown by the significantly similar tissue distributions of proline oxidase, Δ^1 -pyrroline-5-carboxylate dehydrogenase and glutamate dehydrogenase. Reverse metabolism of glutamate or proline to ornithine would be atypical in rat tissues with these distributions of unidirectional enzyme reactions.

The directions and relative rates of the metabolic conversions in animal tissues between ornithine, proline and glutamate (Stetten & Schoenheimer, 1944) remain uncertain. It has been assumed that glutamate is converted into proline (Adams, 1970) and proline into ornithine (Smith *et al.*, 1967), but a net transformation (from glutamate to citrulline) has been demonstrated only in small intestine (Windmueller & Spaeth, 1974, 1976). Relative activities of three unidirectional enzymes acting on or forming Δ^1 -pyrroline-5-carboxylate will determine in part this metabolic flow in each tissue, since Δ^1 -pyrroline-5-carboxylate is a common intermediate among the three compounds. A fourth enzyme acting on Δ^1 -pyrroline-5-carboxylate, the reversible ornithine aminotransferase (L-ornithine-2-oxo acid aminotransferase, EC 2.6.1.13), has been studied with regard to its tissue distribution and hormonal controls in rats (Herzfeld & Knox, 1968; Herzfeld & Greengard, 1969). The formation of proline by Δ^1 -pyrroline-5-carboxylate reductase [L-proline-NAD(P)⁺ 5-oxidoreductase, EC 1.5.1.2] (Meister *et al.*, 1957; Smith & Greenberg, 1957; Adams & Goldstone, 1960b; Peisach & Strecker, 1962), of glutamate by Δ^1 -pyrroline-5-carboxylate dehydrogenase (L-pyrroline-5-carboxylate-NAD⁺ oxidoreductase, EC 1.5.1.12) (Strecker, 1960b; Adams &

Goldstone, 1960c; Brunner & Neupert, 1969), and of Δ^1 -pyrroline-5-carboxylate by proline oxidase (Johnson & Strecker, 1962; Kramar, 1967; Taggart & Krakauer, 1949) have been described primarily in livers of rat and calf. A limited tissue distribution was determined only for the first of these enzymes (Meister *et al.*, 1957).

Previous studies of these enzymes were hampered by impure preparations of DL- Δ^1 -pyrroline-5-carboxylate (Strecker, 1960a). The properties of the enzymes were therefore reinvestigated by using pure synthetic DL- Δ^1 -pyrroline-5-carboxylate (Mezl & Knox, 1976). Characteristics previously not described were found for each of the enzymes, some of which may participate in the physiological control of their activities. The distribution in rat tissues of these enzymes, and of ornithine aminotransferase and glutamate dehydrogenase [L-glutamate-NAD⁺ oxidoreductase (deaminating), EC 1.4.1.2], indicates that there are widespread and independently distributed tissue capacities for the synthesis of proline from ornithine, and for the oxidation of proline to α -oxoglutarate, but not for the formation of ornithine from glutamate.

Materials and Methods

DL- Δ^1 -Pyrroline-5-carboxylate was obtained by periodate oxidation of DL-hydroxylysine and standardized by the weight of its dinitrophenylhydrazone

* To whom reprint requests should be sent.

† Present address: Department of Genetics, University of Hawaii, Honolulu, HI 96822, U.S.A.

(Mezl & Knox, 1976). After quantitative decomposition of the hydrazone, the DL- Δ^1 -pyrroline-5-carboxylate was kept only for a few days in 0.25 M-HCl at 4°C and neutralized to pH 6.8 just before use. NADH (Calbiochem, La Jolla, CA, U.S.A.) was prepared fresh daily as a 26 mM solution in 0.05 M-potassium phosphate buffer, pH 7.2. L-5-Oxoproline (L-pyrrolidone-5-carboxylate) was obtained from Fluka (Buchs, Switzerland), *o*-aminobenzaldehyde from K & K Laboratories (Plainview, NY, U.S.A.) and other reagents were from standard sources. Glutamate decarboxylase (*Escherichia coli*) was from Sigma (St. Louis, MO, U.S.A.) and was demonstrated to be specific for L-glutamate.

Amino acid analysis was carried out at 33°C on a Beckman model 120C instrument by using sodium citrate buffer (50 ml/h) adjusted to pH 3.18 with HCl (Benson & Patterson, 1965).

Acid-ninhydrin determination of proline and Δ^1 -pyrroline-5-carboxylate

Our modification of the Chinard (1952) reaction took advantage of the increased stability of the coloured complex when salted out (Bergman & Loxley, 1970), and used a preliminary nitrosation followed by acid hydrolysis to destroy non-proline compounds (Goodwin, 1972). The sequential reactions were carried out in a single, disposable, capped polyethylene tube. Deproteinized tissue extracts containing 0.02–0.15 μ mol of proline or its equivalent in 0.25 ml were combined with 0.5 ml of conc. HCl and 0.25 ml of 2.5 M-NaNO₂. Standards were added to tissue extracts. Water (0.25 ml) replaced the nitrite solution in samples not to be nitrosated. After 10 min at room temperature (25°C), 0.15 ml of 4.8 M-NH₄Cl was added. The tubes were covered with marbles and heated for 20 min in a boiling-water bath. After cooling, the mixtures were neutralized by the addition of 0.4 ml of 10 M-NaOH, 2.5 ml of a mixture containing 0.10 M-ninhydrin and 2.14 M-NaH₂PO₄ and H₃PO₄. The tubes, covered with marbles, were again heated for 60 min in a boiling-water bath, when the red product was formed and salted out. This was extracted into 3.0 ml of benzene by shaking the capped tubes for 15 min on a mechanical shaker. *A*₅₂₀ was read in a Zeiss PMQ II spectrophotometer. A larger volume of benzene was needed if more than 0.15 μ mol of proline was to be analysed in a sample.

With nitrosation, only proline produced significant amounts of colour in this reaction. Without nitrosation, cysteine (Gaitonde, 1967), ornithine and proline (Goodwin, 1972), and Δ^1 -pyrroline-5-carboxylate formed colour. Proline was measured as a routine in this way after nitrosation, and Δ^1 -pyrroline-5-carboxylate by the difference in its colour with and without nitrosation. Millimolar absorbance coeffi-

cients determined in tissue extracts (zero-time blanks) for proline and Δ^1 -pyrroline-5-carboxylate respectively were 17.4 and 11.9 without nitrosation, and 17.1 and 0.15 with nitrosation. Colour production from standards was about 10% greater in the absence of tissue extracts.

Animal tissues

Kx syngeneic rats (MRC Laboratory Animal Centre registration, formerly NEDH strain) were killed between 09:30 and 11:00 h and the tissues were analysed immediately. Tissues not otherwise identified were from male adults (80–100 days old). Gestational ages were determined by the foetal weights (Stotsenburg, 1915). The weights of mammary glands during lactation were corrected for milk content by determinations of lactose in the homogenates (Slater, 1957). Tumours from various sources and with different growth rates were transplanted into appropriate strains of rats and analysed during their exponential phase of growth. They included mammary carcinoma DMBA 5A, rhabdomyosarcoma NS 104, Morris hepatoma 7777 and Morris renal-cell carcinoma MK-1 (Machovich & Greengard, 1972). Rat submaxillary-gland tumours (Cataldo & Shklar, 1964) were provided by Dr. C. C. Cappuccino (Cappuccino & Shklar, 1976). Fresh calf liver was obtained from a local abattoir.

Tissues were disintegrated in a glass/Teflon homogenizer with 9 vol. of 0.25 M-sucrose containing 0.05 M-potassium phosphate, pH 6.8. Particulate preparations were obtained by centrifugation at 15000g for 15 min. These steps were carried out at 25°C for preparations to be analysed for Δ^1 -pyrroline-5-carboxylate reductase, and otherwise at 0°C. Preparations for the assay of Δ^1 -pyrroline-5-carboxylate reductase were kept at 25°C in the presence of 2.5 mM-mercaptoethanol. For Δ^1 -pyrroline-5-carboxylate dehydrogenase assays, the homogenate or particulate preparation was treated with 0.5% Triton X-100 at 0°C for 30 min, and centrifuged at 20000g for 20 min. The supernatant fraction contained all activity, but the enzyme was not truly solubilized. The Triton X-100 treatment completely inhibited proline oxidase. Necessary dilutions of the tissue extracts were made immediately before assay. All enzyme assays were completed within 4 h from preparations of the tissue.

Enzyme assays

Reactions were run under optimum conditions in which the rates were linear with time for the period used and proportional to the enzyme concentrations. Results are expressed as units (μ mol of product/min) per g of tissue.

Δ^1 -Pyrroline-5-carboxylate reductase. Reaction mixtures consisted of 40mM-potassium phosphate buffer, pH 6.8, 4.4mM-DL- Δ^1 -pyrroline-5-carboxylate, 2.0mM-NADH and diluted tissue extract in a total volume of 0.75ml. The components were equilibrated at 37°C before addition of the enzyme, then incubated for 5 min in a shaking water bath at 37°C. The reaction was stopped by the addition of 0.2ml of 50% (w/v) trichloroacetic acid. Substrate-free blanks and ones containing standard amounts of proline or DL- Δ^1 -pyrroline-5-carboxylate were deproteinized at zero time. Duplicate samples (0.25ml) of the filtrates were analysed by the acid-ninhydrin method with nitrosation for the proline formed.

Δ^1 -Pyrroline-5-carboxylate dehydrogenase. Reaction mixtures contained 3.7mM-NAD⁺, 1.6mM-DL- Δ^1 -pyrroline-5-carboxylate, 90mM-potassium phosphate buffer, pH 7.5, and 0.1ml of diluted tissue extracts in a total volume of 1.0ml. The formation of NADH at 37°C was measured at 340nm in a Gilford recording spectrophotometer. Substrate-free blank activities were subtracted from the experimental values.

Proline oxidase. The method was modified from that described by Johnson & Strecker (1962) in which the Δ^1 -pyrroline-5-carboxylate formed was determined at the end of the reaction with *o*-aminobenzaldehyde (Mezl & Knox, 1976). Reaction mixtures contained 53mM-potassium phosphate buffer, pH 8.0, 158mM-L-proline, 1.6 μ M-cytochrome *c* and the extract of up to 20mg of tissue in a total volume of 1.9ml. These were incubated at 37°C with shaking in air. The reaction was stopped after 30min with 1.0ml of 10% (w/v) trichloroacetic acid. The Δ^1 -pyrroline-5-carboxylate formed was determined by the addition of 0.1ml of 0.2M-*o*-aminobenzaldehyde, centrifugation and reading the A_{440} in the supernatant fraction after 30min, by using the millimolar absorbance coefficient of 2.58 (Mezl & Knox, 1976).

Results

Cold-sensitivity of Δ^1 -pyrroline-5-carboxylate reductase in rat tissues

Δ^1 -Pyrroline-5-carboxylate reductase of rat liver that had been cooled below room temperature during or after its preparation was nearly inactive when later warmed for assay. The loss was essentially complete and irreversible on exposure of homogenates or soluble fractions to 0°C for 30min (Fig. 1). The enzyme was not protected against subsequent cold inactivation by prior dialysis or by the addition of its coenzyme or of thiol compounds. Combinations of active and cold-inactivated preparations had activity equal to the sum of their separate activities. The enzyme could be partially protected against subsequent cold inactivation by a prior heat treatment

(60°C for 8 min), which was the maximum at which the activity survived (Table 1). The heat-labile non-diffusible factor that was responsible for the cold inactivation was not equally distributed in all tissues; in some tissues the enzyme was only partially inactivated by cold treatment. Calf liver was the tissue used in some earlier studies (Adams & Goldstone, 1960b; Peisach & Strecker, 1962) and its enzyme was not inactivated by exposure to cold (Table 1).

Fig. 1. Effect of cold on the activity of Δ^1 -pyrroline-5-carboxylate reductase

Fresh 10% (w/v) liver homogenates prepared at 25°C were kept at 25°C (----) or 0°C (—) with (○) or without (Δ) addition of 2.5mM-mercaptoethanol. Samples were removed at intervals, diluted with medium at 37°C and assayed at 37°C. Points are means of three experiments; bars indicate 1 s.d.

Table 1. Temperature effect on Δ^1 -pyrroline-5-carboxylate reductase activity of pretreated tissue extracts

Results are averages from two or more rat tissue homogenates made at 25°C, preheated (60°C for 8 min) as indicated, and then kept at 25°C or 0°C for 30min before being assayed at 37°C. Supernatant fractions were tested after the heat treatment.

		Activity (μ mol/min per g)			
		Not preheated		Preheated (60°C)	
Kept 30 min at	...	25°C	0°C	25°C	0°C
Rat liver		31.5	1.2	29.2	23.4
Rat small intestine		28.5	1.0	13.0	2.5
Rat kidney		23.0	9.3	19.4	15.5
Rat hepatoma 7777		40.0	12.0	25.6	—
Calf liver		42.3	45.7	—	—

Table 2. *Stoichiometry and products of the Δ^1 -pyrroline-5-carboxylate dehydrogenase reaction*

Triton X-100 extracts of rat and calf livers were incubated for 10 min with 5 mM-NAD⁺ and the listed amounts of DL- Δ^1 -pyrroline-5-carboxylate. After the reactions stopped, all could be started again by the addition of more substrate. NADH was measured optically and other reactants were determined by amino acid analysis of HClO₄ filtrates. For the changes measured, reactants consumed have negative signs, those produced have positive signs. γ -Aminobutyrate derived from glutamate was measured in a sample of the neutralized HClO₄ filtrate by incubation for 30 min with 2.3 units of L-glutamate decarboxylase (Najjar, 1957).

		DL- Δ^1 -Pyrroline-5-carboxylate added (μ mol)	Measured changes (μ mol)				
Liver (mg)			Δ^1 -Pyrroline-5-carboxylate	NADH	Glutamate	Proline	γ -Amino-butyrate
Rat	0.25	0.15	—	+0.07	—	—	—
	0.25	0.25	—	+0.12	—	—	—
	2.50	1.75	-0.88	+0.85	+0.82	0.0	+0.75
Calf	5.0	0.20	—	+0.09	—	—	—
	5.0	0.40	—	+0.22	—	—	—
	10.0	2.04	-0.63	+0.63	+0.49	+0.19	+0.46
					+0.68		

The properties of the Δ^1 -pyrroline-5-carboxylate reductase in rat liver, previously determined from the residual activity in cold-treated preparations, were restudied in our more active preparations without finding significant discrepancies. The colorimetric measure of proline provided a way to assay Δ^1 -pyrroline-5-carboxylate reductase quantitatively in crude tissue extracts and to establish that the activity was wholly in the soluble tissue fraction. Activity with NADPH at any concentration was less than one-tenth that with NADH. The apparent K_m for NADH was 0.58 mM, midway between the reported values of 0.24 mM (Smith & Greenberg, 1957) and of 0.84 mM (Peisach & Strecker, 1962) and too high for convenient continuous measurement of the zero-order reaction. The K_m for L- Δ^1 -pyrroline-5-carboxylate was 0.62 mM, somewhat higher than the reported values of 0.21 mM (Smith & Greenberg, 1957) and of 0.33 mM (Peisach & Strecker, 1962). A variety of thiol reagents inhibited the enzyme, consistent with the presence of a sensitive thiol group (Smith & Greenberg, 1957). Inhibition by the products (proline and NAD⁺) was less than previously reported (Peisach & Strecker, 1962), and was insufficient to slow the reaction under the assay conditions or to account for its irreversibility. Equivalence of the amounts and rates at which Δ^1 -pyrroline-5-carboxylate and NADH were consumed and proline formed, and the consumption of only half of the DL- Δ^1 -pyrroline-5-carboxylate in the presence of excess of enzyme, confirmed the known stoichiometry and stereospecificity of the reaction (Meister *et al.*, 1957; Adams & Goldstone, 1960a). The irreversibility of the reaction of Δ^1 -pyrroline-5-carboxylate reductase (Meister *et al.*, 1957; Johnson & Strecker, 1962) was confirmed under a large variety of conditions.

Stereospecificity of Δ^1 -pyrroline-5-carboxylate dehydrogenase

Both isomers of earlier impure DL- Δ^1 -pyrroline-5-carboxylate preparations were reported to be oxidized to the expected amounts of DL-glutamate by Δ^1 -pyrroline-5-carboxylate dehydrogenase of ox liver (Strecker, 1960b). D-Glutamate, known to be an impurity in these substrate preparations (Strecker, 1960a) was qualitatively identified in the product formed by the enzyme in insect flight muscle (Brosemer & Veerabhadrappe, 1965). The ox liver enzyme did oxidize L-3-hydroxy- Δ^1 -pyrroline-5-carboxylate to L-hydroxyglutamate (Adams & Goldstone, 1960c). We found that reactions with excesses of either rat or calf liver enzyme stopped when half of our pure DL- Δ^1 -pyrroline-5-carboxylate was consumed, except in an experiment with 10 mg of the less-active calf liver preparation, in which only about 30% was metabolized (Table 2). The amounts of NADH and glutamate formed were equivalent to the Δ^1 -pyrroline-5-carboxylate that disappeared. Agreement was not exact with the calf liver preparation, which formed some proline. In both rat and calf liver preparations the glutamate formed was identified as the L-isomer by its quantitative conversion into γ -aminobutyric acid by L-glutamate decarboxylase.

Other properties of Δ^1 -pyrroline-5-carboxylate dehydrogenase were as previously described, with minor exceptions. Its reaction with NAD⁺ was about 10 times faster than with the same concentrations of NADP⁺. The apparent K_m values for NAD⁺ of the calf liver (0.16 mM) and rat liver (0.30 mM) enzymes were about 5 times those previously reported (Adams & Goldstone, 1960c; Strecker, 1960b). The rate was maximal between pH 6.7 and 7.7, although more alkaline pH optima have been described (Strecker,

Table 3. *Inhibition of proline oxidase from liver by certain tissue extracts*

Particle preparations equivalent to the given weights of rat liver and other tissues were assayed alone or in combination for proline oxidase activity as described in the Materials and Methods section. Foetal small intestine was from day 20 to gestation. Pretreatments of particle preparations included dialysis for 2h against 0.05M-phosphate, pH6.8, or heating as indicated.

Particle preparations assayed		Δ^1 -Pyrroline-5-carboxylate formed in assay (μ mol)
Liver (mg)	Other tissue (mg)	
2.5	—	0.375
0.0	Pancreas: 2.5	0.0
2.5	0.5	0.099
2.5	2.5	0.008
2.5	Pancreas, dialysed 2h: 2.5	0.0
2.5	Pancreas, 60°C for 10min: 2.5	0.018
2.5	Pancreas, 100°C for 5 min: 2.5	0.320
0.0	Small intestine: 2.5	0.0
2.5	0.5	0.236
2.5	2.5	0.146
0.0	Foetal small intestine: 5.0	0.208
2.5	5.0	0.592
0.0	Lung: 5.0	0.010
2.5	5.0	0.380

1960b; Adams & Goldstone, 1960c). The reaction occurred twice as fast in phosphate or Tris buffer as in other organic buffers tested. The enzyme was extremely sensitive to inhibitions by *p*-hydroxymercuribenzoate and *N*-ethylmaleimide, and there were potent competitive inhibitions by L-hydroxyproline and L-5-oxoproline as reported (Strecker, 1960b; Adams & Goldstone, 1960c). The apparent K_m for Δ^1 -pyrroline-5-carboxylate was 0.18mM-L-isomer. The enzyme did not oxidize glyceraldehyde and valeraldehyde (Adams & Goldstone, 1960c; Peisach & Strecker, 1962), in confirmation of an earlier report.

The reaction of Δ^1 -pyrroline-5-carboxylate dehydrogenase was not reversible under a variety of conditions (Meister *et al.*, 1957; Smith *et al.*, 1967). After incubation with L-glutamate and NADH, Δ^1 -pyrroline-5-carboxylate was not detectable by sensitive chemical analyses and under these conditions active Δ^1 -pyrroline-5-carboxylate reductase in preparations made at 25°C did not trap any Δ^1 -pyrroline-5-carboxylate as proline. L-5-Oxoproline was unreactive in the reverse reaction.

Assay of proline oxidase

We observed an optimum pH of 8.0 and assays were made at this pH instead of 7.4 as in earlier studies

(Johnson & Strecker, 1962; Kramar, 1967; Taggart & Krakauer, 1949) and phosphate buffer was used, in which the reaction was twice as fast as in some organic buffers tested. The conditions used were unfavourable for other reactions, and particulate preparations of liver formed the amounts of Δ^1 -pyrroline-5-carboxylate expected from the proline that disappeared. Inhibition of Δ^1 -pyrroline-5-carboxylate dehydrogenase with 10 μ M-*p*-hydroxymercuribenzoate left the proline oxidase active without increasing the yield of Δ^1 -pyrroline-5-carboxylate. Further metabolism of added DL- Δ^1 -pyrroline-5-carboxylate (0.3mM) did not occur significantly under assay conditions.

Because proline oxidase activity was confined to only a few tissues, combinations of extracts from active and inactive tissues were made to test the validity of the assay in the latter tissues. Extracts from adult pancreas, and to a lesser degree those from small intestine, inhibited the enzyme from liver. The inhibitor present in the extracts was not diffusible and it was destroyed by boiling (Table 3). Pancreas and small intestine taken from fetuses and postnatal rats up to 2 weeks old had proline oxidase activity and did not inhibit other active tissue extracts. Lung and other tissues without activity in themselves did not show this inhibition.

Tissue distributions of Δ^1 -pyrroline-5-carboxylate reductase, Δ^1 -pyrroline-5-carboxylate dehydrogenase and proline oxidase

Activities of each of the enzymes in a series of rat tissues are given in Table 4. Except for the values for proline oxidase in the two tissues where an inhibitor prevented measurement of this enzyme (values marked †), the values should be proportional to the concentration of enzyme. Each enzyme was characteristically distributed with at least a 10-fold range of activity between the different tissues. High Δ^1 -pyrroline-5-carboxylate reductase activity was present in many tissues. Δ^1 -Pyrroline-5-carboxylate dehydrogenase activity was also widespread, but with a relatively high value only in a few tissues, namely liver, kidney, adrenal glands and heart. These tissues also contained proline oxidase. The latter activity was the lowest of the three enzymes and confined to only a few tissues.

The patterns of distribution of the individual enzymes conformed to two known types. Δ^1 -Pyrroline-5-carboxylate reductase was relatively high in all foetal and tumour tissues, being higher in the foetal heart, brain and lung than in the corresponding adult organs, and higher in hepatoma 7777, mammary carcinoma 5A and rhabdomyosarcoma NS 104 than in the parent tissues of these tumours. This distribution is typical of the 'foetal-type' enzymes whose genetic control resides in that hierarchy of

Table 4. *Enzyme distributions in rat tissues*

Values represent average activities from three or more independent determinations. The coefficients of variation (s.d./mean) of each type of assay were less than 0.25 (Δ^1 -pyrroline-5-carboxylate reductase), 0.10 (Δ^1 -pyrroline-5-carboxylate dehydrogenase) and 0.15 (proline oxidase). Tissues not measured are marked with a dash.

Tissue	Activity ($\mu\text{mol/min per g of tissue}$)		
	Δ^1 -Pyrroline-5-carboxylate reductase	Δ^1 -Pyrroline-5-carboxylate dehydrogenase	Proline oxidase
Foetal			
Liver	42.3	6.0	0.18
Small intestine	28.1	0.0	1.22
Kidney	25.5	1.15	0.26
Lung	24.8	0.27	—
Brain	23.6	0.12	0.02
Heart	23.0	0.46	0.07
Neoplastic			
Hepatoma 7777	40.4	0.04	0.05
Rhabdomyosarcoma NS 104	27.8	—	—
Submaxillary tumour	23.5	0.58	0.0
Mammary carcinoma 5A	20.5	—	—
Renal MK-1	10.5	3.3	0.0
Adult			
Submaxillary gland	56.8	0.39	0.0
Liver	33.1	33.5	5.29
Lactating (15-day) mammary gland*	29.5	0.75	—
Small intestine	27.5	0.38	0.02†
Kidney	23.8	14.5	2.04
Thymus	20.1	0.28	—
Spleen	19.4	0.42	0.0
Brain	15.4	0.40	0.42
Pancreas	10.3	0.90	0.0†
Lung	6.9	0.39	0.05
Colon	6.6	0.32	—
Heart	6.6	2.80	0.50
Epididymis	6.1	0.80	—
Skeletal muscle	5.1	0.44	0.0
Adrenal gland	5.0	4.5	0.17
Pregnant mammary gland	5.0	0.75	—

* Milk-free mammary-gland weights were determined by lactose measurements (Slater, 1957).

† Assay prevented by inhibitor in tissue.

genes expressed in immature tissues and differentiated neoplasms (Knox, 1976). The other two enzymes (Δ^1 -pyrroline-5-carboxylate dehydrogenase and proline oxidase) were low in or absent from foetal and tumour tissues, and were present with relatively high activities only in certain adult tissues. This distribution is typical of those 'adult-type' enzymes controlled by a second hierarchy of genes expressed in postnatal life and in certain adult tissues. Their presence or absence chemically differentiates adult tissues from one another. An exception was the relatively high value of proline oxidase in foetal small intestine, an organ in which an inhibitor of this enzyme developed later.

The degrees of 'coprominence' in tissues of the several enzymes was appraised objectively by correla-

tions between their ranked tissue activities (Snedecor, 1948). Published analyses in the same tissues of ornithine aminotransferase (Herzfeld & Knox, 1968; Herzfeld & Raper, 1976*a,b*; Knox, 1976) and glutamate dehydrogenase (Herzfeld, 1972; Herzfeld & Raper, 1976*a,b*; Knox, 1976) were included in the comparisons. There were significant degrees of similarity between the tissue distributions of some of these enzymes (Table 5). Ornithine aminotransferase and Δ^1 -pyrroline-5-carboxylate reductase formed one cluster [$R_s = 0.467$ (Spearman rank correlation coefficient; Snedecor, 1948); $P < 0.05$]. Submaxillary glands, for example, contained high activities of both these enzymes as well as arginase (Herzfeld & Raper, 1976*b*), and had a high potential for proline synthesis from arginine. The same three enzymes

Table 5. 'Coprominences' in rat tissues of enzymes concerned with ornithine and proline metabolism

Tissues in Table 4 were ranked by their contents of each enzyme, and the tissue rankings for each enzyme correlated with those for every other enzyme by Spearman's rank correlation (Snedecor, 1948). Analyses of rat tissues for ornithine aminotransferase (Herzfeld & Knox, 1968; Herzfeld & Raper, 1976a,b; Knox, 1976) with the new determination of 0.28 unit/g in skeletal muscle, and analyses for glutamate dehydrogenase (Herzfeld, 1972; Herzfeld & Raper, 1976a,b; Knox, 1976) with the new determinations (units/g) of 2.7 in foetal small intestine, 0.05 in skeletal muscle, 3.0 in adrenal gland and 0.05 in mammary gland of pregnant rat (day 19) were included in the comparisons. Correlation coefficients are tabled, with the number of paired tissues for which there were analyses in parentheses. Coefficients significantly different from zero (*t*-test) are indicated by superscripts (**P* < 0.05; †*P* < 0.01).

	Ornithine aminotransferase	Δ^1 -Pyrroline-5- carboxylate reductase	Proline oxidase	Δ^1 -Pyrroline-5- carboxylate dehydrogenase
Δ^1 -Pyrroline-5-carboxylate reductase	0.467* (23)	1.00	—	—
Proline oxidase	0.125 (18)	0.239 (20)	1.00	—
Δ^1 -Pyrroline-5-carboxylate dehydrogenase	-0.072 (17)	-0.171 (20)	0.354 (20)	1.00
Glutamate dehydrogenase	0.145 (21)	0.194 (22)	0.585* (14)	0.663† (15)

increased co-ordinately in lactating mammary gland, and in this tissue an actual metabolic flow from arginine to proline (and glutamate) was demonstrated *in vitro* and *in vivo* (Mezl & Knox, 1977). A second cluster of enzymes with significantly similar tissue distributions comprised proline oxidase, Δ^1 -pyrroline-5-carboxylate dehydrogenase and glutamate dehydrogenase. These had mutual correlations (R_s) of 0.354, 0.585 and 0.663 (Table 5). Given the unidirectional nature of the first two steps, this indicated a potential pathway for proline and Δ^1 -pyrroline-5-carboxylate degradation to glutamate and α -oxoglutarate in certain tissues. There were no significant similarities between the distributions of enzymes of the first and second clusters. Submaxillary gland was rich in those of the first cluster and poor in those of the second cluster, but in some tissues both potentials were relatively high. Liver, kidney, brain and foetal small intestine, for example, contained significant amounts of both Δ^1 -pyrroline-5-carboxylate reductase and proline oxidase, which, if uncontrolled, could wastefully recycle proline.

Discussion

The new finding, that Δ^1 -pyrroline-5-carboxylate reductase in crude rat tissue extracts was inactivated by cold, made it possible to obtain at least 10 times greater tissue activities by avoiding cold treatment. Properties of the fully active enzyme were, however, generally the same as those that had been described from the lower activity in cold-treated tissue extracts.

The degree of inactivation by cold was more complete for Δ^1 -pyrroline-5-carboxylate reductase than for other enzymes with this sensitivity (Pullman *et al.*, 1960; Scrutton & Utter, 1965; Havar *et al.*, 1965; Jarabak *et al.*, 1966). The possible re-activation of the cold-treated enzyme was slight in relation to its original activity, but a severalfold increase had been

observed when a chilled rat liver enzyme fraction was kept at room temperature for 2 h (Smith & Greenberg, 1957). Inactivation in the cold did not appear to be an intrinsic property of Δ^1 -pyrroline-5-carboxylate reductase, but was due to a heat-labile, non-diffusible inhibitor which exerted its full effect over 30 min and occurred in various amounts in different tissues of the rat but not in calf liver. Cold-lability of the enzyme in human tissues has since been demonstrated; in both rat and human lung more of the inhibitor was present in foetal tissues than in their adult counterparts (Greengard & Herzfeld, 1977). Such an inhibitor that is separable from the enzyme could be analogous to an antienzyme (Heller *et al.*, 1976) and could provide a mechanism for controlling the rate of proline synthesis in tissues.

A relatively high activity of Δ^1 -pyrroline-5-carboxylate reductase in hepatomas compared with liver (Peraino & Pitot, 1962) was confirmed, and its prominence demonstrated also in other kinds of tumours. Predictably (Knox, 1976), it was also relatively high in foetal compared with adult tissues. These two characteristics are shared by a number of enzymes regulated by one of the two recognized hierarchies of genetic control (Knox, 1976).

Δ^1 -Pyrroline-5-carboxylate dehydrogenase was re-defined as a stereospecific enzyme that acted in both rat and calf liver only on the L-isomer of our pure DL- Δ^1 -pyrroline-5-carboxylate. Interference in its assay by Δ^1 -pyrroline-5-carboxylate reductase was minimal in rat tissues, because that enzyme was removed from particulate preparations through its solubility and its inactivation by cold. Except for its higher apparent K_m with NAD⁺, the properties of Δ^1 -pyrroline-5-carboxylate dehydrogenase otherwise resembled those originally described for it (Strecker, 1960b; Adams & Goldstone, 1960c). Though its absolute activity was less in many tissues than that of Δ^1 -pyrroline-5-carboxylate reductase, the low acti-

vities were apparently physiologically significant. With 0.75 unit/g in lactating mammary gland, where Δ^1 -pyrroline-5-carboxylate reductase was 49 units/g, this tissue produced from ornithine 2 or 3 times as much glutamate as proline (Mezl & Knox, 1977).

Proline oxidase activity was absent from many tissues, and in two of these without demonstrable activity (small intestine and pancreas) a heat-labile non-diffusible inhibitor was present that could prevent any activity from being manifested. The inhibitor appeared after birth in these tissues, and was similar in this respect to an inhibitor of asparagine synthetase found in many postnatal tissues (Huang & Knox, 1975).

The three enzyme activities measured all catalysed unidirectional reactions that make or use L- Δ^1 -pyrroline-5-carboxylate. Two are mitochondrial and respond to the adult type of genetic enzyme regulation (Δ^1 -pyrroline-5-carboxylate dehydrogenase and proline oxidase). One is soluble and cold-labile and responds to the foetal type of genetic enzyme regulation (Δ^1 -pyrroline-5-carboxylate reductase). They are differently distributed among the tissues, but the Δ^1 -pyrroline-5-carboxylate reductase capacity correlates with that of ornithine aminotransferase in the same tissue; this indicates that there is a widespread potential among tissues for proline synthesis from ornithine. The significant correlations of the activities of proline oxidase, Δ^1 -pyrroline-5-carboxylate dehydrogenase and glutamate dehydrogenase with one another indicate a real but less widespread potential for proline oxidation in rat tissues. In a few tissues both of these potentials co-exist, and in these some means of control of the separate reactions that make and remove proline would appear to be needed. The cold-inactivation of Δ^1 -pyrroline-5-carboxylate reductase by a protein-like inhibitor present in different concentrations in different tissues might represent such a means of control. The same three enzymes also catalyse analogous reactions with hydroxyproline as well as with proline derivatives (Adams & Goldstone, 1960b,c), and this additional metabolic function may also affect the enzyme distributions among tissues.

The three unidirectional enzyme reactions measured here orient the potential metabolic flow in various tissues from ornithine to proline or from proline to glutamate. A flow to form ornithine would appear to be distinctly atypical, because proline oxidase activities are limited and are not significantly associated with ornithine aminotransferase activities in the same tissues, and because there is no detectable conversion of glutamate into Δ^1 -pyrroline-5-carboxylate by the reversal of the Δ^1 -pyrroline-5-carboxylate dehydrogenase reaction. However, in rat small intestine Windmueller & Spaeth (1974, 1976) demonstrated that glutamate was converted into citrulline. The route and the enzymes responsible

for this transformation are not known. This tissue contains relatively high amounts of ornithine aminotransferase (Herzfeld & Raper, 1976a) as well as Δ^1 -pyrroline-5-carboxylate reductase, but reduction of glutamate to Δ^1 -pyrroline-5-carboxylate remains problematical in this and other animal tissues.

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References

- Adams, E. (1970) *Int. Rev. Connect. Tissue Res.* **5**, 1-92
- Adams, E. & Goldstone, A. (1960a) *J. Biol. Chem.* **235**, 3492-3498
- Adams, E. & Goldstone, A. (1960b) *J. Biol. Chem.* **235**, 3499-3503
- Adams, E. & Goldstone, A. (1960c) *J. Biol. Chem.* **235**, 3504-3512
- Benson, J. V. & Patterson, J. A. (1965) *Anal. Biochem.* **13**, 265-280
- Bergman, I. & Loxley, R. (1970) *Anal. Chem.* **42**, 702-706
- Brosemer, R. W. & Veerabhadrapa, P. S. (1965) *Biochim. Biophys. Acta* **110**, 102-111
- Brunner, G. & Neupert, W. (1969) *FEBS Lett.* **3**, 283-286
- Cappuccino, C. & Shklar, G. (1976) *Arch. Oral Biol.* **21**, 147-152
- Cataldo, E. & Shklar, G. (1964) *J. Dent. Res.* **43**, 568-569
- Chinard, F. P. (1952) *J. Biol. Chem.* **199**, 91-95
- Gaitonde, M. K. (1967) *Biochem. J.* **104**, 627-633
- Goodwin, J. F. (1972) *Clin. Chem.* **18**, 449-453
- Greengard, O. & Herzfeld, A. (1977) *Cancer Res.* **37**, 884-891
- Havir, E. A., Taimir, H., Ratner, S. & Warner, R. C. (1965) *J. Biol. Chem.* **240**, 3079-3088
- Heller, J. S., Fong, W. F. & Cannelakis, E. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1858-1862
- Herzfeld, A. (1972) *Enzyme* **13**, 246-251
- Herzfeld, A. & Greengard, O. (1969) *J. Biol. Chem.* **244**, 4894-4898
- Herzfeld, A. & Knox, W. E. (1968) *J. Biol. Chem.* **243**, 3327-3332
- Herzfeld, A. & Raper, S. M. (1976a) *Biochim. Biophys. Acta* **428**, 600-610
- Herzfeld, A. & Raper, S. M. (1976b) *Enzyme* **21**, 471-480
- Huang, Y. Z. & Knox, W. E. (1975) *Enzyme* **19**, 314-328
- Jarabak, J., Seeds, A. E. & Talalay, P. (1966) *Biochemistry* **5**, 1269-1279
- Johnson, A. B. & Strecker, H. J. (1962) *J. Biol. Chem.* **237**, 1876-1882

- Knox, W. E. (1976) *Enzyme Patterns in Fetal, Adult and Neoplastic Rat Tissues*, 2nd edn., Karger, Basel
- Kramar, R. (1967) *Enzymologia* **33**, 33–37
- Machovich, R. & Greengard, O. (1972) *Biochim. Biophys. Acta* **286**, 375–381
- Meister, A., Radhakrishnan, A. N. & Buckley, S. D. (1957) *J. Biol. Chem.* **229**, 789–800
- Mezl, V. A. & Knox, W. E. (1976) *Anal. Biochem.* **76**, 430–440
- Mezl, V. A. & Knox, W. E. (1977) *Biochem. J.* **166**, 105–113
- Najjar, V. A. (1957) *Methods Enzymol.* **3**, 462–466
- Peisach, J. & Strecker, H. J. (1962) *J. Biol. Chem.* **237**, 2255–2260
- Peraino, C. & Pitot, H. C. (1962) *Biochim. Biophys. Acta* **62**, 585–587
- Pullman, M. E., Penefsky, H. S., Datta, A. & Racker, E. (1960) *J. Biol. Chem.* **235**, 3322–3329
- Scrutton, M. C. & Utter, M. F. (1965) *J. Biol. Chem.* **240**, 3714–3723
- Slater, T. F. (1957) *Analyst (London)* **82**, 818–825
- Smith, A. D., Benziman, M. & Strecker, H. J. (1967) *Biochem. J.* **104**, 557–564
- Smith, M. E. & Greenberg, D. M. (1957) *J. Biol. Chem.* **226**, 317–327
- Snedecor, G. W. (1948) *Statistical Methods*, Iowa College Press, Ames, Iowa
- Stetten, M. R. & Schoenheimer, R. (1944) *J. Biol. Chem.* **153**, 113–132
- Stotsenburg, J. M. (1915) *Anat. Rec.* **9**, 667–682
- Strecker, H. J. (1960a) *J. Biol. Chem.* **235**, 2045–2050
- Strecker, H. J. (1960b) *J. Biol. Chem.* **235**, 3218–3223
- Taggart, J. V. & Krakauer, R. B. (1949) *J. Biol. Chem.* **177**, 641–653
- Windmueller, H. G. & Spaeth, A. E. (1974) *J. Biol. Chem.* **249**, 5070–5079
- Windmueller, H. G. & Spaeth, A. E. (1976) *Arch. Biochem. Biophys.* **175**, 670–676