Effect of β-Aminopropionitrile (Lathyrus Factor) on Free Hydroxyproline, Proline and Glycine in Chick Embryo.* (24985)

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(Introduced by L. Dienes)
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Chick embryos treated with beta-aminopropionitrile, the lathyrus factor, become stunted, develop gross skeletal deformities(1,2), histological changes in aorta, a startling degree of tissue fragility(3) and increased extractability of collagen(4). Alterations in behavior and properties of collagen of the lathyritic chick will be reported in detail elsewhere. Among acid-soluble, low molecular-weight constituents extractable from chick embryos are free amino acids including hydroxyproline(5). The latter is considered unique to collagen in the animal kingdom. Concentration of this amino acid rises in the brain of embryos treated with cortisone(6) although no explanation has been advanced. It is present in free form in negligible amounts in postnatal tissues, the currently held explanation being that proline is hydroxylated to hydroxyproline only after incorporation of the former into a polypeptide chain(7). This may not necessarily be the case in embryonic tissues, specifically those of the chick. There is a progressive decrease of free hydroxyproline in the chick brain with time and it is absent in adult birds(8). This study compares concentrations of total free amino acids, hydroxyproline, proline and glycine in brains and carcasses of normal and lathyritic chick embryos at 2 different periods of development. These 3 amino acids were chosen because of their particular prominence in amino acid constitution of collagen.

Methods. Fertile hen eggs were incubated under controlled conditions of temperature and humidity. On 4th day of incubation 0.32 mg (LD_{50}) of beta-aminopropionitrile fumarate (BAPN)§ in 0.1 ml of saline was injected onto the chorio-allantoic membrane. Injected and non-injected batches of eggs were harvested on 10th and 17th day. Two separate and complete experiments were run for 10th day and 3 for 17-day embryo groups. Only living embryos showing typical stigmata of lathyrism (stunting, parrot beaks and bowed tibiae), were used. About 20 normal and 20 experimental embryos were processed in each experiment. They were decapitated while living, the carcasses rapidly weighed, chopped finely with scissors and homogenized with 2 volumes of ice cold 0.6 M perchloric acid. Brains were rapidly removed, weighed and plunged into liquid nitrogen. After pulverization in chilled steel mortar, the powder was homogenized with 2 volumes of 0.6 M perchloric acid for 2 minutes at about 5°C. In Exp. D half the brains were rapidly frozen in CO_2 snow instead of liquid nitrogen. Homogenates were cleared by centrifugation (59,000 x g, 30 minutes) and supernatant solutions were neutralized with KOH in the cold. Potassium perchlorate crystals were filtered off at 0°C and the resulting neutral extract was stored frozen. Analyses on whole extracts for glycine(9), hydroxyproline(10), proline(11), and α-amino acids with a ninhydrin method(12) were carried out before and after hydrolysis of the extracts. Hydrolysis was performed in 6 N hydrochloric acid in sealed tubes in oil bath at 138° for 3 hours. The results are recorded as "free" (result before hydrolysis) and "bound" (the difference between results before and after hydrolysis). Aliquots of extracts were freed of salt over a Dowex 50 column and examined by 2 dimensional paper chromatography in 2 different solvent systems(13 and Deffner personal

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TABLE I. Acid-Extractable Amino Acids of 10-Day Chick Embryos.

	μM/g wet wt							
	Brain				Carcass			
	Normal		Lathyritic		Normal		Lathyritic	
Exp. No.	A	B	A	B	A	B	A	B
Ninhydrin*								
Free	18.9	20.5	16.5	23.4	13.4	16.6	12.6	13.5
Bound	3.7	14.3	8.4	12.8	3.5	9.9	2.9	7.7
Hydroxyproline								
Free	.13	.17	.14	.20	.11	.14	.11	.13
Bound	.0	.02	.0	.04	.01	.05	.0	.04
Proline								
Free	.27	.36	.37	.43	.25	.28	.26	.26
Bound	.07	.14	.20	.16	.15	.13	.16	.08
Glycine								
Free	.51	.64	.53	.62	.62	.46	.59	.34
Bound	4.16	2.57	3.84	2.83	3.03	2.14	2.48	1.69
Pro/Hypro	2.1	2.1	2.6	2.1	2.3	2.0	2.4	2.0
Gly/Hypro	3.9	3.8	3.8	3.1	5.6	3.3	5.4	2.6

* Reported as μM leucine color equivalence.

communication) and stained with ninhydrin or isatin primarily to determine relative intensities of proline and hydroxyproline spots.

Results. Analytical data are tabulated in Tables I and II, the former referring to 10-day embryos and the latter to 17-day embryos. There are, at 10 days, no striking differences between extracts from normal and lathyritic embryos in any parameters examined, despite the fact that treated embryos (Table I) showed all morphological manifestations of lathyrism as well as greatly increased tissue fragility. The ratios of proline to hydroxyproline and glycine to hydroxyproline are essentially the same for normal and lathyritic animals in both brain and carcass. There is practically no bound hydroxyproline in extracts from any of the tissues, in contrast to proline, where there is a significant amount, and particularly to glycine where bound amino acid accounts for the bulk of total extracted. The free hydroxyproline, proline, and glycine concentrations in brain extracts are only slightly if at all higher than in extracts of the carcass.

Extracts from 17-day embryos, in sharp contrast to those described above, revealed significant differences in proportions of free hydroxyproline and proline in both brain and carcass (Table II). There is a significant increase of free hydroxyproline in the lathyritic brain over that in the normal, and a parallel decrease in free proline, the latter diminution also being clearly demonstrated in the carcass. In contrast there is an increase in amount of free glycine over 10-day embryo in both normal and lathyritic tissues. Thus, there is a reversal of the ratio of free proline to free hydroxyproline in the lathyritic animal whereas glycine to hydroxyproline ratio stayed essentially constant. Both features are manifested in the carcass as well as in the brain.

It is worthy of note that there is essentially no bound hydroxyproline extractable in perchloric acid as compared with proline and glycine, suggesting that there are little if any free small peptides or other complexes of hydroxyproline in the tissue.

Paper chromatograms run simultaneously on normal and lathyritic extracts and stained with isatin confirmed the rise in free hydroxyproline and the fall in proline.

Discussion. Reciprocal changes in free proline and hydroxyproline in the 17-day lathyritic chick embryo suggest an increased rate of conversion of the former to the latter rather than degradation of collagen to amino acids. The work of Mitoma *et al.*(14) strongly suggests that, in the chick embryo, hydroxylation of free proline does occur. This postulate leaves the sharp rise in free glycine, which comprises 30% of amino acid residues

TABLE II. Acid-Extractable Amino Acids of 17-Day Chick Embryos.

	μM/g wet wt							
	Brain						Carcass	
	Normal			Lathyritic			Normal	Lathyritic
Exp. No.	C	D	E	C	D	E	C	C
Ninhydrin*								
Free	24.3	25.7	18.5	20.8	22.8	17.6	16.5	13.8
Bound	17.8	14.8	13.1	15.1	15.0	14.8	15.2	12.2
Hydroxyproline								
Free	.31	.32	.22	.44	.42	.57	.33	.37
Bound	.02	.02	.0	.0	.0	.0	.12	.06
Proline								
Free	.41	.32	.52	.30	.27	.32	.44	.23
Bound	.20	.20	.17	.25	.25	.0	.45	.38
Glycine								
Free	.83	.95	.50	1.30	1.12	1.27	1.08	1.26
Bound	2.57	3.06	2.44	2.22	2.81	2.09	2.05	.84
Pro/Hypro	1.3	1.0	2.4	.68	.64	.56	1.3	.62
Gly/Hypro	2.7	3.0	2.3	3.0	2.7	2.2	3.3	3.4

* Reported as μM leucine color equivalence.

of collagen, unexplained. If these changes do represent collagen degradation, proline must be rapidly reutilized in other pathways in contrast to the other 2 amino acids.

Since the amino acid changes are first observed long after structural changes are well defined, it may be concluded (a) that the former are due to a lathyrus factor effect on mechanisms which are not detectable until late in maturation of the embryo and (b) that the agent may exert its deforming effect along other pathways. The fact that similar free hydroxyproline increases unaccompanied by skeletal deformities have been noted after cortisone administration favors these hypotheses.

Studies of the free amino acid patterns in similarly deformed embryos produced with 6-aminonicotinamide(15) or semicarbazide (16) will be of interest.

Summary. Analyses of brain and carcass of normal and lathyritic chick embryos for free hydroxyproline, proline, glycine, and total ninhydrin reactive material are reported. At 10 days development, 6 days after BAPN administration, no obvious alterations in these parameters were found although embryos were grossly malformed. At 17 days of development, 13 days after treatment, a reversal in ratios of proline to hydroxyproline was found; proline concentration fell, and that of hydroxyproline rose in both brain and carcass of lathyritic embryos as compared to the normals. A rise in free glycine concentration resulted in unchanged ratio of glycine to hydroxyproline.

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