ALTERATIONS IN STATE OF MOLECULAR AGGREGATION OF COLLAGEN INDUCED IN CHICK EMBRYOS BY β-AMINOPROPIONITRILE (LATHYRUS FACTOR)*

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Dramatic malformations of mesenchymal tissues may be induced in a variety of growing animals by a series of simple compounds (lathyrogenic agents) exemplified by β-aminopropionitrile (BAPN) (1–4).

The skeletal and vascular deformities have been best described in the rat (5–10). Marked periosteal new bone formation and exostosis of the long bones, kyphoscoliosis, hernias, and dissection of the aorta are the most typical alterations (11–13). Lesions are localized to the connective tissues; some authors implicate the ground substance, using as evidence histochemical staining for mucopolysaccharides (7, 14–17) and analysis for hexosamine (18), others focusing on collagen (11, 19–22) and elastin (6, 14, 23–25). Conflicting reports concerning tissue concentrations of these constituents in the lathyritic state are numerous. Some investigators have obtained evidence suggesting decrease or cessation of collagen (11, 26, 22) and mucopolysaccharide (11, 18) synthesis and others have claimed no change in collagen (18, 21) or mucopolysaccharide (27, 13). Microscopic changes in the cells of affected tissues are described (10).

One of the striking characteristics of lathyritic animals is weakening of tendinous and ligamentous attachments, epiphyseal plates, skin, cartilage, and healing wounds (8, 28–30, 26), in addition to marked deformations of the skeleton. Our early studies on BAPN-treated chick embryos, (31, 32) in which gross morphologic changes had been previously described (3, 32 *a*), revealed a dramatic loss of tensile strength of the mesenchymal tissues associated with increased solubility of connective tissue components including collagen. Clemmons (33) simultaneously reported increased extractibility of collagen from croton oil pouches in BAPN-treated rats. The ability

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of BAPN to induce the appearance of neutral salt extractible collagen in the skin of mature and aging guinea pigs has been described by the authors (34).

This report is concerned mainly with the state of aggregation of collagen in the lathyritic chick embryo as indicated by its extractibility and properties in solution, plus possible correlations with the changes in tensile properties of the whole animal.

Methods

General Procedures.—Fertilized eggs of the white Leghorn variety were injected *via* the chorioallantoic membrane with β-aminopropionitrile fumarate (BAPN)[1] in 0.1 ml. of sterile distilled water through a small hole drilled into the shell, the hole subsequently being covered with scotch tape. A small air hole was made in one end of the egg prior to injection. Eggs were incubated at 37°.

The bulk of the experiments reported here were performed on eggs incubated for 14 days prior to injection and harvested at 17 days. In other experiments the agent was introduced at 4 days with harvesting at 10 and 17 days of incubation.

The effect of dose on mortality was determined by applying graded amounts of the agent to the eggs and LD_{50} was determined.

Normal and treated embryos of all groups were fixed in neutral formalin and also in cold methanol. Tissues were dehydrated, cleared, and embedded in paraffin in the usual manner and sections of various organs and tissues including skin, bone, and aorta in particular were stained with hematoxylin and eosin, van Gieson, Verhoeff's elastic, Gomori-Bielchowsky reticulin, PAS, and toluidine blue. Whole embryos were cleared and stained with alizarin (35).

"Fragility" Measurements.—Measurements of change in tensile properties of the whole living embryo were performed by determining the time necessary to separate the head from the body under a constant stretching load. It was found that the value in normal embryos was a function of the weight of the animal. The load used varied somewhat with each batch of eggs. Preliminary trials on normal embryos were run to establish the load required to provide an average breaking time between 50 and 150 seconds. Normal 17 day embryos usually required a 500 gm. weight. Ten to twenty embryos were used for each measurement and the weight of each whole embryo recorded. Fragility was determined both as a function of dose of BAPN and time elapsed after administration of the agent.

Analytical Methods.—Skin, bone, and aorta were dissected from the fresh living embryo in ice-cooled trays. Skin was carefully freed of feathers; bone and cartilage were freed of muscle and tendon. Tibiae and femora were examined separately in several experiments and found to give similar results. The major aortic branches were dissected and freed from the heart. All tissues were weighed wet, then dried in a vacuum desiccator over phosphorus pentoxide, transferred to weighed hydrolysis tubes, and dried to constant weight again in the Abderhalden apparatus *in vacuo* at 110° for 16 hours. They were then analyzed for the following constituents: nitrogen (36), hydroxyproline (37), proline (38), and tyrosine (39).

Both dried tissues and tissue extracts were hydrolyzed in sealed tubes in an oil bath at 138° for 3 hours in 6 N HCl for analysis for proline and hydroxyproline. In the hydroxyproline analysis a blank omitting peroxide was always used to account for possible interference from other pyrroles (40). Alkaline hydrolysis was used for the estimation of tyrosine.

Extraction Procedures.—Fresh wet tissues—skin, bone rudiments, and aortae pooled from six to twelve embryos—were minced finely with scissors in an ice-cooled tray and extracted

[1] BAPN, β-aminopropionitrile, kindly provided by the Abbott Laboratories, North Chicago.

with shaking at 5°C. for 24 hours in two volumes (V/W) of 1 M NaCl buffered with phosphate, pH 7.6, ionic strength 0.02. Extracts were separated from residues by centrifugation in the Spinco model L preparative ultracentrifuge at 100,000 *g* for 1 hour. The cleared supernatant fluids were then passed through fine sintered glass filters in the cold.

Physical Chemical Procedures.—The relative viscosity of all extracts was determined at 5°C. in Ostwald viscometers, flow time of 60 seconds at this temperature. Reduced viscosity as a function of concentration was determined for purified collagen isolated from skin and bone extracts. Many of the extracts were examined in the Spinco model E analytical ultracentrifuge at 56,000 R.P.M. at 5°C. using a wedge cell for double determinations in most cases. Some extracts were treated with purified collagenase from *Clostridium histolyticum* (provided through the generosity of Dr. Paul Gallop), followed by sedimentation analyses.

Specific optical rotation was determined for purified collagen isolated from extracts of lathyritic chick tissue in 0.5 per cent acetic acid at 5°C. in a water jacketed 2 decimeter cell in a Rudolph photoelectric polarimeter using the sodium D line.

Purification of Collagen in the Extracts.—Separate skin and bone extract pools were prepared from a number of BAPN-treated embryos and the collagen precipitated by the addition of an equal volume of cold 5 M NaCl solution (41). After 16 hours at 5°C, the precipitate was removed by centrifugation and redissolved in phosphate buffer, pH 7.6 ionic strength 0.5. After 24 hours the viscous solution was centrifuged at 100,000 *g* for 1 hour to remove a very small insoluble residue. The clear solution was then fractionated as described previously (42) for saline extracts of guinea pig skin. The final purified collagen precipitate was redissolved in 0.5 per cent acetic acid to produce a clear viscous solution which could then be stored in the refrigerator for several months. Samples of these solutions were dialyzed against acetic acid with repeated changes, then lyophilized for chemical analysis. Other samples were used for the determination of intrinsic viscosity, specific optical rotation, and sedimentation constant extrapolated to zero concentration at 5°C.

RESULTS

Injection at 14 Days, Harvesting at 17 Days of Incubation

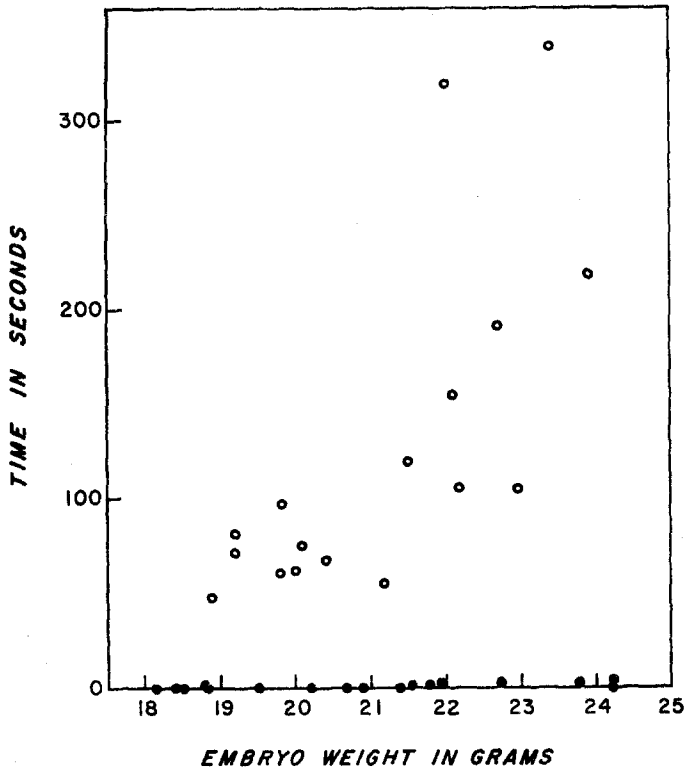
There is an obvious dosage dependence of mortality with the LD_{50} at about 20 mg. Only viable embryos were used. In control experiments nearly 100 per cent of the embryos survived the injection of water, saline, or insulin.

Gross Appearance of Treated Embryos.—Embryos injected with 20 mg. BAPN at 14 days and examined at 17 days of incubation were usually indistinguishable in general appearance and size from the normal 17 day group. However, their extreme fragility was manifested on handling; tendons, cartilages, and muscles detached with great ease. The skin was thinner and much more fragile than normal. The alizarin-stained skeleton revealed considerable thickening of femora and tibiae with dislocations of joints, separation of epiphyses from shaft, and often nearly complete rotation of the tibia. Fig. 1 illustrates the alizarin-stained skeleton of a normal 17 day embryo (Fig. 1 *b*) as compared with a 17 day treated embryo (Fig. 1 *a*).

Histology.—Few histological differences between the mesenchymal tissues of normal and treated embryos were noted. No changes were observed in the aorta; the dermis of the treated embryos appeared distinctly thinner in certain areas than the corresponding normal dermis. The tibiae despite their obvious

increase in shaft width seen in the alizarin-stained lathyritic embryos, showed little histologic difference from the normal.

Measurement of Fragility of Embryos.—As a measurement of the change in tensile properties of the embryo, determinations were made of the time it required to separate the head from the body under the tension of a constant



TEXT-FIG. 1. Fragility of embryos measured by time required to separate heads from bodies under a constant stretching load of 500 gm. as a function of embryo weight. Embryos injected with 20 mg. of BAPN at 14 days and examined at 17 days of incubation.

load on the neck. It was not feasible to measure the breaking point as a function of load because of the long-range extensibility of the neck. It is evident that this parameter is far from a simple index of tensile strength and could depend upon many factors. However, consistent and usable data were obtained in this manner which could be related directly to the influence of the agent. It is evident from Text-fig. 1 which is a plot of the data obtained from normal 17 day embryos and from those injected at 14 and examined at 17 days of incubation that there is a uniform increase in rupture time with increase in weight for the normal embryos. The lathyritic group of the same weight distribution

exhibited far greater fragility, the heads separating from the body almost immediately under the same load. Histological examination revealed that the site of the rupture took place uniformly between the 3rd and 4th cervical vertebra in both normal and lathyritic.

In order to present the data in simpler fashion a "Fragility Index" was used as a measure of change in tensile properties of treated embryos as compared to a group of controls examined simultaneously. The value:

$$\frac{\text{Average time of rupture (seconds)}}{\text{Average weight of embryos (grams)}} \times 10,$$

using ten to twenty normal animals and the same number of treated animals, proved to be a useful and reproducible index for comparative purposes. Fragility

TABLE I

Dosage Effect on Embryo Fragility, and Extractibility of Collagen from Bone. Injection at 14 Days, Examination at 17 Days; 500 Gm. Load Used for "Fragility Index"

Dose	Average weight of embryos	Fragility index	Extract hydroxyproline	η rel
mg.	*gm.* $\pm \sigma$		*μg./ml.*	
0.0	21.6 ± 1.2	38	27	2.3
0.5	21.7 ± 2.9	43	—	—
2.0	21.9 ± 2.6	5	97	6.0
10.0	—	—	276	51.0
20.0	22.8 ± 2.6	0	630	150.0

σ, standard deviation of weight of 12 embryos in each group.

of treated embryos was found to be dependent upon dosage and time elapsed after injection. Effect of dosage is shown in Table I. Effect of the passage of time after injection is illustrated in Text-fig. 5.

Analyses of Tissues.—As seen in Table II the water content decreased with age in both skin and bone and was somewhat higher in the lathyritic tissues. No difference was noted in the aortae. Apart from the normal increase with age no significant difference in hydroxyproline content between normal and lathyritic tissues was observed. In the lathyritic aorta, however, this amino acid was significantly diminished. Proline content was the same for normal and lathyritic tissues except for aorta in which again there was a significant diminution in the lathyritic. Tyrosine content was not consistently altered in the lathyritic tissues.

Extraction of Tissues.—Pronounced changes were observed in 1 M extracts of bone, skin, and aorta of lathyritic animals as compared with those of normal tissues. They were extremely viscous and had high concentrations of hydroxyproline. The ultracentrifuge pattern revealed a slow moving, hypersharp boundary which was eliminated on treatment with collagenase. Protocols of a repre-

sentative experiment are shown in Text-fig. 2. Extract viscosity and hydroxyproline content were dependent upon dosage as indicated in Table I. Relative viscosity and hydroxyproline were always determined on all extracts since the former measurement provides confidence that the hydroxyproline is present in the usual highly asymmetric type of collagen molecule (42). Extractible collagen appeared within 1 hour after administration of the drug and increased steadily with time at least to 72 hours (Text-fig. 3).

It was found that three successive 24 hour extractions in cold 1 M NaCl was sufficient to remove all the soluble collagen. More than 60 per cent of the total

TABLE II

Analysis of Bone, Skin, and Aorta of Normal Controls and Test Embryos Injected with 20 Mg. of BAPN on 14th Day and Analyzed on 17th Day of Incubation

Tissue	Gm. per 100 gm. dry tissue*				
	Dry weight	N	Hypro	Pro	Tyr
Normal skin, 14 days	9.6 ± 0.19	12.0 ± 0.3	0.92 ± 0.13	3.5	2.38
Normal skin, 17 days	12.0 ± 0.67	12.2 ± 0.25	2.18 ± 0.20	4.5	2.17
Lathyritic skin, 17 days	11.2 ± 0.20	12.5 ± 0.10	2.14 ± 0.21	4.3	2.33
Normal bone, 14 days	14.2 ± 0.08	9.4 ± 0.25	1.51 ± 0.30	3.6	1.58
Normal bone, 17 days	21.1 ± 0.54	8.6 ± 0.30	2.32 ± 0.15	3.3	1.52
Lathyritic bone, 17 days	17.9 ± 1.39	8.0 ± 0.15	2.13 ± 0.08	3.4	1.23
Normal aorta, 17 days	10.4 ± 0.15	12.0 ± 0.19	0.92 ± 0.03	3.6	1.99
Lathyritic aorta, 17 days	10.4 ± 0.25	12.6 ± 0.26	0.63 ± 0.08	2.9	1.96

* Mean of three or more determinations ±½ the range. Proline and tyrosine were single determinations (in duplicate).

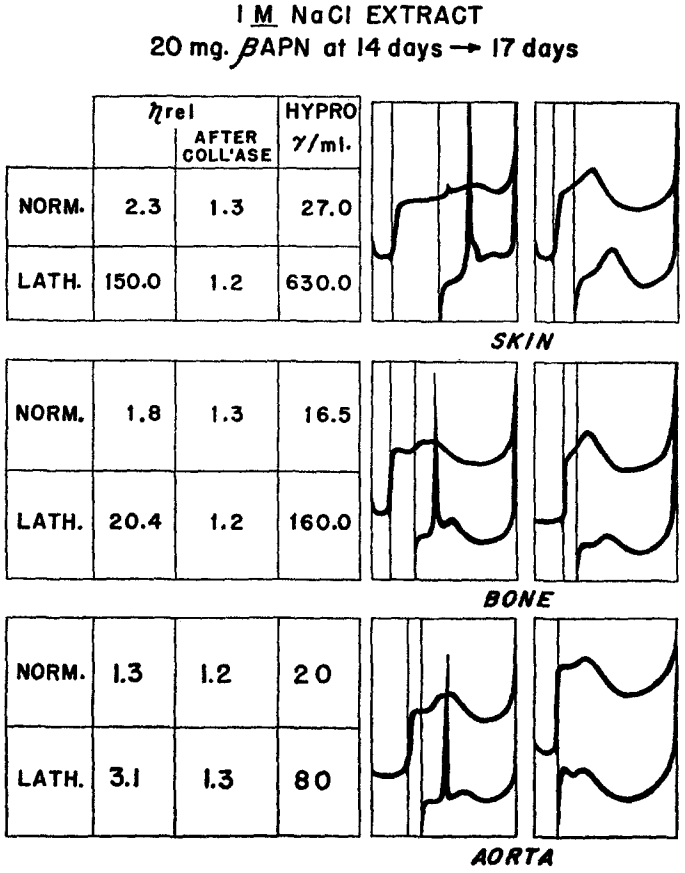
collagen from lathyritic skin, 40 per cent from bone, and 40 per cent from aorta could be extracted with cold 1 M saline while less than 1 per cent could be obtained from comparable normal tissues. Indeed collagen could not be extracted from normal bone and skin with acid citrate buffer or 0.5 per cent acetic acid.

A relatively large amount of collagen could be extracted from lathyritic tissues with cold physiological saline although not nearly as much as with hypertonic NaCl.

The amount of free hydroxyproline in the normal and lathyritic extracts were the same, about 7 μg./ml., accounting for 40 per cent of the total hydroxyproline in a normal skin or bone extract and less than 6 per cent of that of a lathyritic extract.

Separate Extraction of Long Bone Cartilage and Bony Shaft.—Using the long bones freed of periosteum, the cartilaginous portions were separated from the

bony shaft. Cold 1 M NaCl extracts prepared from separate pools of cartilage and shaft were viscous and contained considerable amounts of dissolved collagen

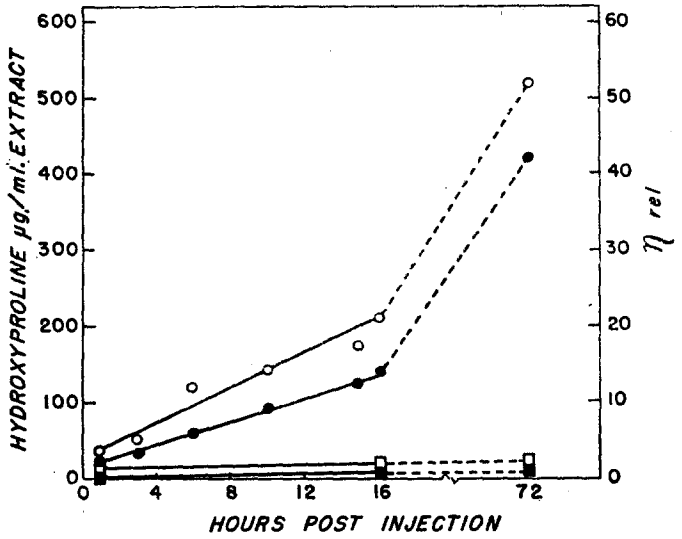


TEXT-FIG. 2. Some characteristics of cold 1 M NaCl extracts of tissues of normal and lathyritic embryos. Tissues extracted 3 days after administration of BAPN. Viscosity, hydroxyproline concentrations, and ultracentrifuge patterns before and after collagenase treatment. Upper patterns are of normal extracts. Right hand set of patterns obtained after collagenase treatment.

(Table III). This is in contrast to extracts of normal bones which contained negligible amounts.

Is Extractible Collagen in Dispersed (Soluble) or in Large Aggregate (Insoluble) Form in Vivo?—If collagen were in molecular dispersion in the ground substance of the lathyritic tissues simple centrifugation of the tissue in absence of extracting medium at room temperature, should yield a clear tissue fluid containing dissolved collagen.

Skin was dissected from normal and lathyritic embryos at room temperature, cut into small bits with scissors, and centrifuged without extracting medium at 100,000 *g* at room temperature for 1 hour. Viscosimetric and hydrox-



TEXT-FIG. 3. Effect of 20 mg. BAPN on extract collagen content as a function of time after administration. Injection at 14 days of incubation. ●, relative viscosity; ○, hydroxyproline concentration of extracts of lathyritic bone; ▮, viscosity; ▯, hydroxyproline concentration of extracts of normal bone.

TABLE III

Extraction of Isolated Cartilage and Bony Shafts from 17 Day Chick Embryos Injected at 14 Days with 20 mg. BAPN

Tissue	η rel*	Hydroxyproline
		µg/ml.*
Normal bone and cartilage	1.5	16.0
Lathyritic cartilage	6.9	96.0
Lathyritic bony shaft	8.3	193.0

* Three consecutive extracts of three volumes of extracting medium V/W, pooled.

yproline analyses of the expressed clear fluids indicated the absence of collagen in the tissue fluids of both the normal and lathyritic embryos. However, if tissues were refrigerated at 5°C. for 24 hours and then centrifuged cold after mincing, again with no extracting medium, the normal tissues behaved as before but the tissue fluid of the lathyritic skin was viscous and contained relatively large amounts of collagen (Table IV).

Thus, the physical state of a portion of the collagen in the lathyritic tissues is temperature-dependent, insoluble at room temperature, soluble in the cold. None of the collagen of the normal animal tissue exhibited this behavior.

Does Extractible Collagen Derive from Old Fibrils?—If the 17 day lathyritic

TABLE IV

Tissue Fluid Obtained by Centrifugation of Whole Skin

	Normal tissue fluid		Lathyritic tissue fluid	
	η rel	Hypro	η rel	Hypro
		γ/ml.		γ/ml.
Warm (37°C)	1.7	48.6	1.7	58.6
Cold (5°C.)	1.5	54.0	2.7*	261.0

* Diluted three times with buffer.

TABLE V

*Hydroxyproline Content of Normal and Lathyritic Skin and Bone before and after Extraction**

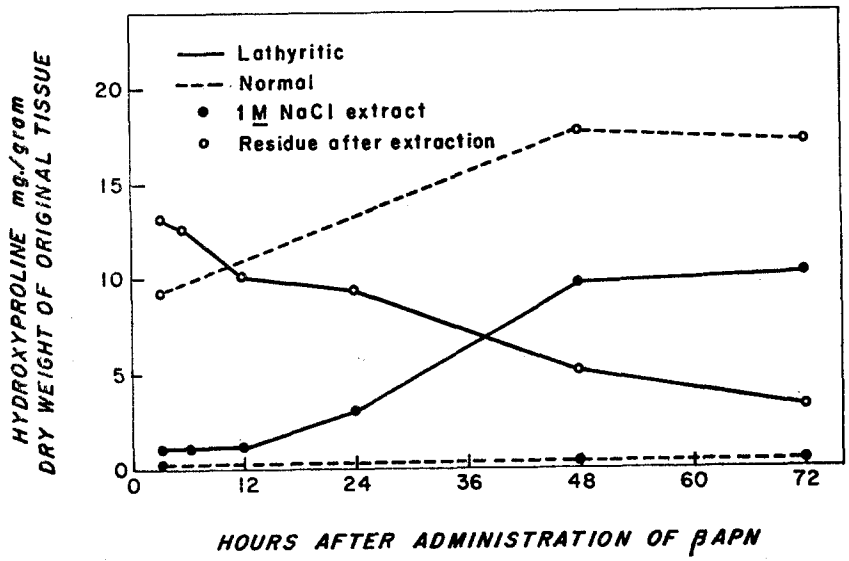
Tissue	μg./gm. dry weight‡					
	14 day normal		17 day normal		17 day lathyritic	
	Total tissue before extraction	Residue after extraction	Total tissue before extraction	Residue after extraction	Total tissue before extraction	Residue after extraction
Skin 1	8.0	9.2	17.2	17.0	14.6	3.4
2	9.2	9.8	21.8	23.3	24.1	5.0
Bone 1	20.8	19.1	26.0	23.3	24.5	14.9
2	19.1	20.9	22.7	21.5	22.7	15.7
3	15.1	18.4	23.2	22.8	21.3	9.4

* Twenty mg. BAPN fumarate injected at 14 days, harvesting at 17 days. Dissected tissues extracted 3 times in cold 1 M saline.

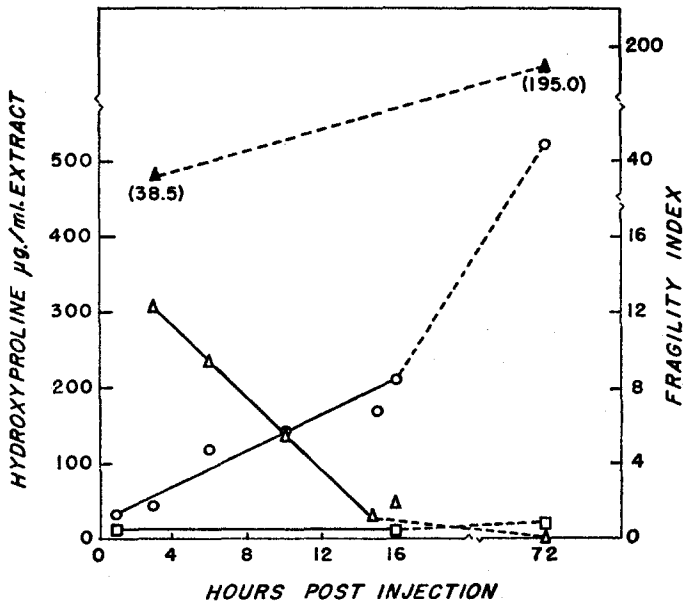
‡ Hydroxyproline concentrations of residues are referred to original dry weight of tissue prior to extraction.

tissue residues (after extraction) contained less collagen than that found at the time of administration of the drug one may conclude that old collagen was solubilized. This indeed proved to be the case as seen from Table V which summarizes the significant values obtained from five such experiments.

Further support of the foregoing data is provided by the inverse relationship between change in extractible collagen in skin and in residue collagen as a function of time, after drug administration, as shown in Text- fig. 4. The same relationship was obtained for bone.



TEXT-FIG. 4. Effect of 20 mg. BAPN on total extractible collagen and residue collagen of skin (after extraction) as a function of time following drug administration.



TEXT-FIG. 5. Effect of 20 mg. BAPN on fragility of embryo and extractible bone collagen concentration as a function of time after administration. ○, hydroxyproline concentration of lathyritic bone extract; □, hydroxyproline concentration of normal bone extract; △, "Fragility Index" of lathyritic embryos; ▲ "Fragility Index" of normal embryos. The lower the index the more fragile is the embryo.

Is There a Relationship between Embryo Fragility and Extractible Collagen?— In several of the experiments described in the previous section "fragility index" was determined on each group of embryos used for tissue extraction at each time interval after administration of the agent. As seen in the protocol in Text-fig. 5 there is a significant increase in fragility within 3 hours after administration of the drug which progresses steadily with increasing extractibility of collagen. The normal animals on the other hand get progressively stronger with time and essentially no collagen is extractible.

Is There Evidence for Alteration of the Molecules of Extracted Collagen?— Fractionation of bone and skin extracts from lathyritic 17 day chick embryos yielded collagen fractions homogeneous by sedimentation and electrophoresis.

TABLE VI

Some Characteristics of Purified Collagen Extracted from Lathyritic Chick Embryo Skin and Bones

	Skin	Bone
Glycine	23.4 gm./100 gm.	24.0 gm./100 gm.
Hydroxyproline	12.9 " " "	11.7 " " "
Proline	9.8 " " "	8.5 " " "
$[\eta]$	—	13.1
$S_{c\to o}$	—	3.2
$\alpha]_D$	−400°	−368°

Purified samples were dissolved in 0.5 per cent acetic acid at 5°C. Concentration was based on dried weight in solution and hydroxyproline measurements.

$[\eta]$, intrinsic viscosity; $S_{c\to o}$, sedimentation coefficient extrapolated to 0 concentration; $\alpha]_D$, specific optical rotation.

In both instances the boundaries were hypersharp and slowly migrating. Specific optical rotation, intrinsic viscosity, and sedimentation coefficient (extrapolated to zero concentration) at 5°C. in 0.5 per cent acetic acid were determined. Glycine, hydroxyproline, and proline content were measured on the salt-free lyophilized and vacuum-dried material (Table VI). It was not possible to obtain a sample of purified collagen from normal chick embryo tissues since these were not at all soluble in either cold neutral salt solutions, acid citrate buffer (pH 3.5), or dilute acetic acid.

The specific optical rotation, intrinsic viscosity, and sedimentation constant are essentially similar to those for dissolved *normal* collagen of other species (43–45, 42). The hydroxyproline, proline, and glycine values are within the range for normal collagens.

Cold samples of lathyritic collagen extracts in 1 M NaCl, pH 6.8–7.6 were warmed to 37°C. in a water bath. Within 15 minutes rigid opaque gels were formed. Examination in the electron microscope revealed large numbers of

normally striated collagen fibrils and amorphous debris. Analysis of supernatant solutions for hydroxyproline indicated that nearly all the collagen had been precipitated. The same experiment performed on purified lathyritic collagen isolated from the crude extract gave the same result.

How Does the Age of Embryo at Time of Application of BAPN Influence the Manifestations of the Lathyritic Process?—Results hitherto reported were from embryos injected with BAPN at 14 days and examined at 17 days of incubation. Batches of embryos were injected at 4 days of incubation *via* the chorio-allantoic membrane with 0.32 mg. BAPN—the LD_{50} at this age—and survivors examined at 17 days of incubation. The resultant skeletal abnormalities (Fig. 1 *c*) included marked antero-posterior bending of tibiae, often to 90°, and bending to a lesser degree of fibulae, femora, and metatarsals; lateral cervical scoliosis and bilateral S-shaped deformities of the mandible were characteristically present. Apart from the bending of the shaft of tibia and fibula, there was no difference histologically between the normal and treated mesenchymal tissues. A comparison of the fragility of treated embryos with normal at 17 days of incubation showed essentially no increased fragility.

TABLE VII

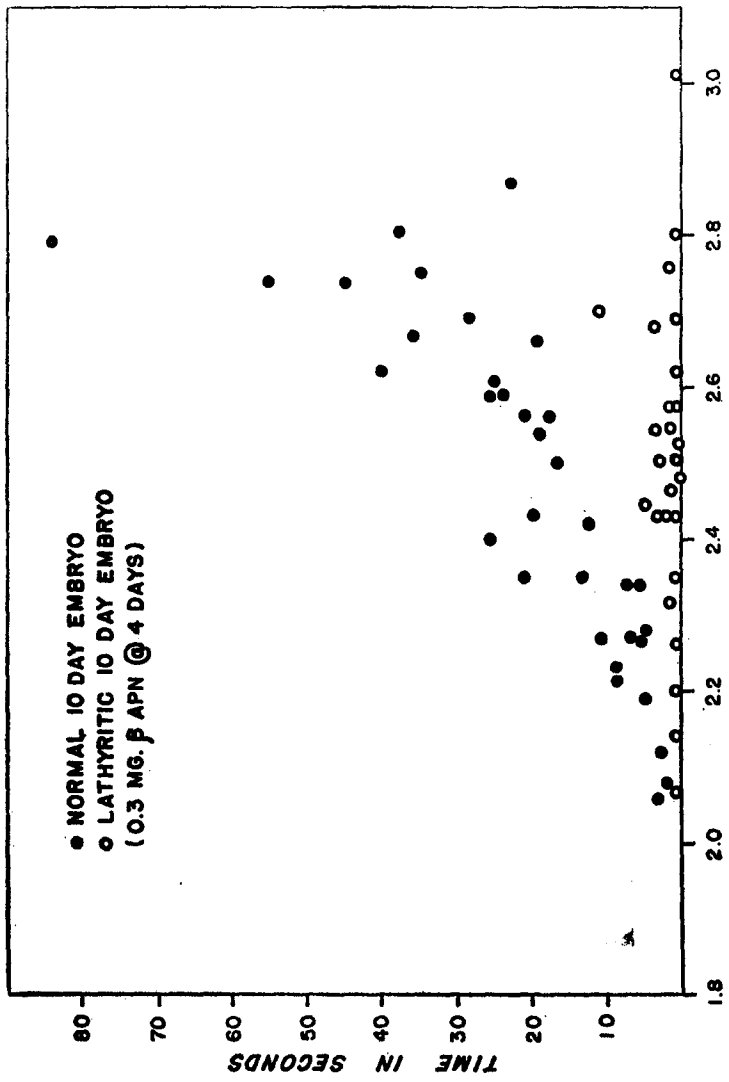
Analysis of Bone and Skin of Normal Controls and Test Embryos Injected at 4 Days and Analyzed at 10 Days of Incubation

Tissue	Gm./100 gm. dry tissue*			
	Dry weight	Nitrogen	Hydroxyproline	Proline
Normal skin	8.23 ± 0.23	13.0	0.39 ± 0.035	2.50
Lathyritic skin	9.27 ± 0.17	10.2	0.36 ± 0.035	2.41
Normal bone	11.14 ± 0.19	11.2	1.38 ± 0.20	2.84
Lathyritic bone	11.50 ± 0.25	11.1	1.53 ± 0.20	3.12

* Mean of three to six determinations ±½ the range. All determinations were done in duplicate.

Cold 1 M NaCl extracts of bone, skin, and aortae from treated embryos were indistinguishable from those of normal tissues—they contained little or no collagen. Sedimentation patterns and viscosity of the extracts were identical.

However, examination of treated embryos at 10 days of incubation, instead of 17 days, revealed in addition to the skeletal deformities previously described, an obvious increase in fragility over the normal (Text-fig. 6). Histological examination showed the significant differences to consist of bending of the cartilaginous rudiment of the tibia, the inner angle of which was buttressed by an exostosis of apparently normal, marrow-containing bone; the aorta, which in the normal at this age was devoid of Hale-staining material, contained a good deal of this substance. Skin and chorioallantois were markedly hemorrhagic. More detailed histological description will be reported elsewhere.



TEXT-FIG 6. Time to rupture neck under constant load (8.5 grams). Fragility of embryos measured by time to separate head from body under a constant stretching load of 8.5 gm. as a function of embryo weight. Four day embryos injected with 0.32 mg. BAPN and examined at 10 days of incubation.

Analyses of skin and bone rudiments indicated a slight diminution in water content and nitrogen in the lathyritic tissues; hydroxyproline and proline were essentially normal (Table VII). Extracts (1 M NaCl) of lathyritic skin and bone rudiments were slightly more viscous and the sedimentation diagram revealed a small, slow moving and poorly diffusing peak not present in the pattern of the normal. Hydroxyproline content although relatively low in both was twice as high in the lathyritic extract.

What Are the Manifestations of a Lathyrogenic Agent, Other Than a Nitrile?—Neuman *et al.* (46) reported the ability of semicarbazide ($H_2NCONHNH_2$) to induce skeletal deformities in the chick embryo, particularly of the beak and tibio-tarsus. These resemble the changes produced by β-aminopropionitrile ($H_2NCH_2CH_2CN$).

TABLE VIII

Effect of Semicarbazide on Embryo Fragility and Extractible Collagen of Bones (Injection at 14 Days, Harvesting at 17 Days)

Dose	Fragility index	η rel	Hypro
mg.			*γ/ml.*
0	6	1.7	32
5	0.5	7.7	117
10	0	11.6	250

Semicarbazide, in doses of 5 and 10 mg. was administered to groups of ten embryos at 14 days of incubation. Examination at 17 days of incubation revealed the increase in fragility and extractible collagen identical with the effect observed with BAPN. The response was dosage-dependent (Table VIII).

Administration of this agent at 4 days of incubation with examination at 10 days, showed the characteristic skeletal deformities, and also increased fragility of the embryo.

DISCUSSION

The results of this study demonstrate a disturbance of intermolecular aggregation within the collagen fibril induced by lathyrogenic agents. The data indicate that a significant proportion of the fibrils formed prior to treatment with BAPN are altered so that they become soluble in cold saline. In the normal chick embryo collagen is not solubilized by cold neutral salt solutions nor by acid buffers. These conclusions have been confirmed by electron microscope examination of thin sections of skin of normal and lathyritic chick embryos (van den Hooff, Levene, and Gross, *J. Exp. Med.*, 1959, **110,** No. 6, in press.

Physical-chemical examination of purified collagen extracted from lathyritic tissues suggests that the molecules are essentially intact. The high negative value of specific optical rotation indicates the "normal" degree of α-helical

configuration of the collagen molecule (45). The value of the intrinsic viscosity is characteristic of the high asymmetry ratio of intact collagen molecules in solution as is the hypersharp slow moving boundary in the sedimentation pattern. Finally the possibility of reconstituting typical cross-striated fibrils by warming neutral salt extracts is further strong indication of intactness of the molecules. Subtle changes, however, may have passed undetected by these methods. Assuming the latter is not the case it would appear that intermolecular cross-links, responsible for insolubility of fibrils, have been disrupted. The molecules have been loosened within the fibrils and solubilized without obvious degradation. *In vitro* studies on the time-dependent solubility properties of fibrils precipitated from purified collagen solutions indicate that secondary valence forces and the specific internal organization of the molecule alone can account for the insolubility of old collagen fibrils in both neutral and acid milieu (47, 48). Whether or not intermolecular bonds of higher energy such as amide or ester links might form in the course of time is not known. There is some evidence to suggest the existence of such bonds *within* the molecule (49, 50). Veis and Cohen (51) believe covalent cross-links between molecules are required to explain certain solubility properties of gelatin. If covalent forces do indeed exist between collagen molecules one might then postulate the activity of a hydrolytic enzyme to explain the loosening of intermolecular bonds in lathyritic collagen. If, however, only secondary forces are involved, enzymatic action is unlikely unless one invokes cleavage of critical side chains without affecting the backbone. Studies are now in progress on the action of low concentrations of lathyrogenic agents on the solubility properties of collagen *in vitro*. It can be said at this time that β-aminopropionitrile does not interfere with fibril formation by warming neutral solutions.

It would appear likely that the extractible lathyritic collagen is not dissolved or dispersed in the tissues but rather is in a large aggregate, probably fibrillar, form insoluble at room temperature but dispersed when cooled. Evidence for this contention is derived from those experiments in which the expressed fluids from cold lathyritic tissues were found to contain relatively large amounts of dissolved collagen whereas none was released from warmed tissues. We know from *in vitro* studies that collagen fibrils will dissolve under certain conditions by cooling (47, 48). We suggest that lathyrogenic agents return collagen to a similar temperature-susceptible state. Follis (21), on the other hand, has proposed that BAPN prevents the formation of fibrils from normally synthesized tropocollagen, based on the appearance of fewer fibrils in electron micrographs of fragmented lathyritic cartilage plus a normal hydroxyproline concentration.

Our analytical data provide no evidence for or against continued normal synthesis of collagen in lathyritic tissues. Although the collagen concentration increases with growth as in the normal embryo this effect might be explained equally well by loss of non-collagenous constituents as by increase in collagen.

The question may be settled by isotope incorporation studies. Hurley and Ham (22), by measuring total collagen in entire granulomas and implanted sponges, have evidence suggesting diminished or absent synthesis of collagen in lathyritic rats. If this holds true for the lathyritic chick embryo then all the extractible collagen derives from previously insoluble fibrils laid down prior to drug administration.

The most obvious and dramatic alteration induced by BAPN was the markedly increased fragility. Limbs and head were detached with ease within 72 hours after administration of the agent to the 14 day embryo. Although there was direct correlation between the increase in extractible collagen and the increased fragility a causal relationship is not yet established. It is tempting to assume that the obviously altered association among collagen molecules as evidenced by increasing extractibility would give rise to the decreasing tissue strength. However, it is also possible that localized alterations of non-collagenous components at tendinous and ligamentous attachments could be responsible. Although tensile strength measurements on tendons have not been made yet, it is our distinct "clinical" impression that the tendons of BAPN-treated embryos were swollen and much more readily torn. Reports in the literature of increased friability of skin (29) and healing wounds (26), softness of bone and "fluidity" of cartilage (30, 11) indicate weakness in the substance of the connective tissues.

Although no difference from the normal in free hydroxyproline concentrations was observed in lathyritic tissue extracts of 17 day embryos injected on day 14, a significant increase was found previously in 17 day embryos injected at 4 days of incubation (52).

An interesting observation was the marked skeletal deformities produced in the 17 day embryo following administration of BAPN on the 4th day of incubation. These animals were not fragile nor could any collagen be extracted from their tissues. Seven days earlier, however, in addition to the deformities already existent, the embryos were extremely fragile and some collagen could be extracted. Thus, it would appear that healing of the fragility-producing lesion occurred between the 10th and 17th day. Examination of the deformed tissues at 17 days gave no clue to the active process. Injection of the agent at a later stage (14 days) produced little morphologic change but physical chemical analysis did unearth a significant tissue defect.

SUMMARY

The lathyrogenic agents, β-aminopropionitrile and semicarbizide, when applied to the chorio-allantoic membrane of the chick embryo produced a dramatic increase in fragility of the embryo.

This alteration was not associated with a change in the concentration of collagen, except in aorta, but was accompanied by a sharp increase in the amount of collagen extractible in cold 1 M NaCl from skin, bone, and aorta.

Increase in fragility and extractible collagen began within 3 hours after introduction of the agent and rose steadily for at least 72 hours. Essentially no collagen could be extracted from tissues of normal chick embryos. Both fragility and amount of extractible collagen were dosage- and time-dependent.

It is concluded that the extractible collagen in lathyrism consists of a large proportion of dissolved fibers previously insoluble and formed prior to administration of the agent. The data also suggest that the "lathyritic" collagen *in vivo* is not in molecular dispersion but in an aggregate or fibrillar form. It is dispersed by cooling.

The extracted collagen could be reconstituted to typical striated fibrils *in vitro* and the molecule appeared to be normal in the gross, with regard to asymmetry ratio and intramolecular helical structure.

The evidence at hand suggests that at least one of the defects induced by lathyrogenic agents is an interference with the normal intermolecular cross-linking within the collagen fibril.

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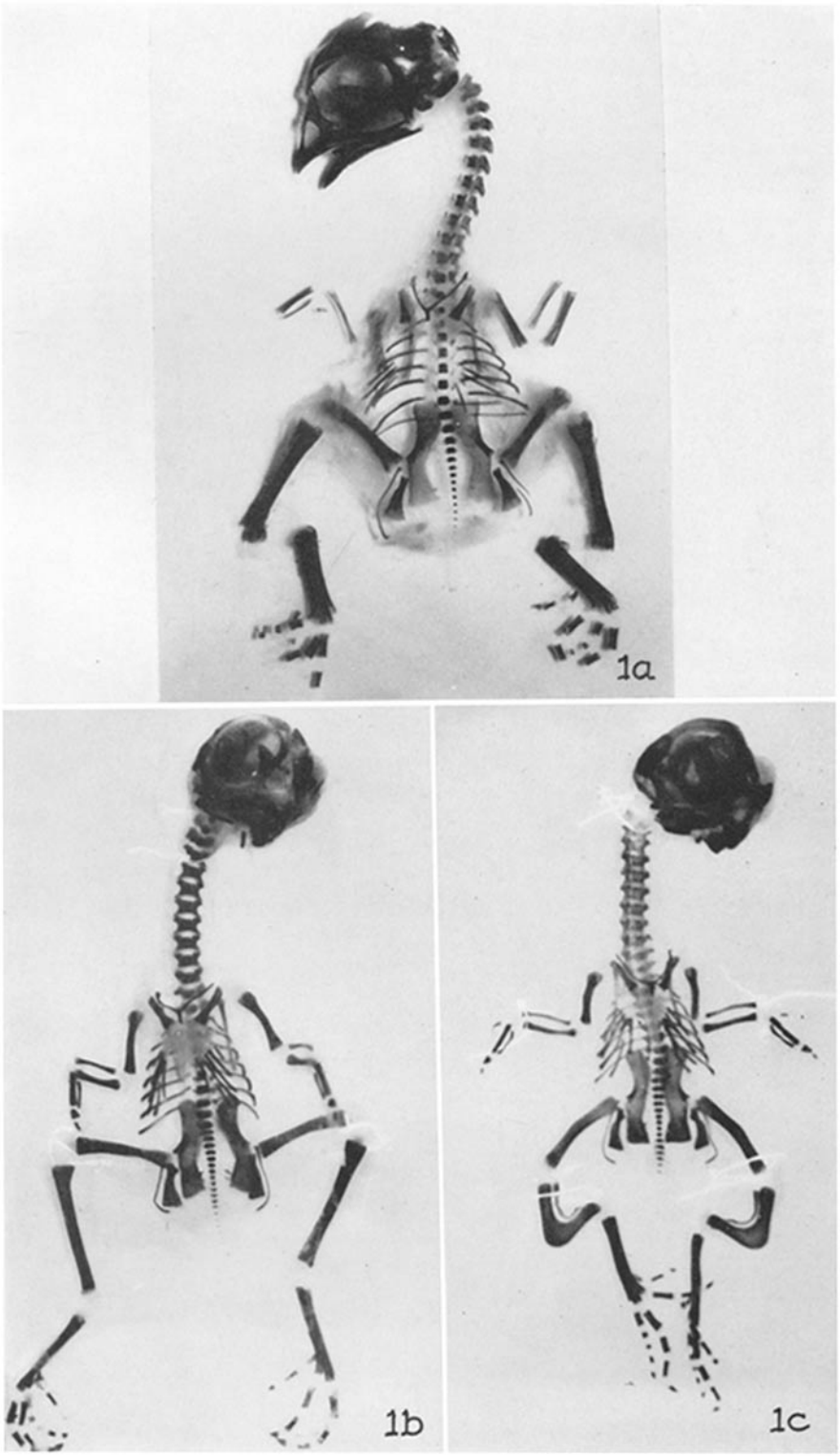
EXPLANATION OF PLATE 65

FIG. 1. Alizarin-stained skeletons of 17 day chick embryos.

FIG. 1 a. Injected at 14 days with 20 mg. of BAPN. Note joint dislocations and lack of bowing of long bones.

FIG. 1 b. Normal control.

FIG. 1 c. Injected at 4 days with 0.32 mg. BAPN. Note extreme bowing of tibiae and fibulae.



(Levene and Gross: Molecular aggregation of collagen)