EXPERIMENTAL LATHYRISM IN THE CHICK EMBRYO

The Distribution of β-Aminopropionitrile*

By SERGE D. ORLOFF,‡ M.D., and JEROME GROSS, M.D.

(From The Department of Medicine, Harvard Medical School and Massachusetts General Hospital, Boston)

(Received for publication, March 6, 1963)

Lathyrogenic agents such as β-aminopropionitrile (βAPN) characteristically induce severe skeletal and other mesenchymal abnormalities in a variety of growing animals (1–3). Associated with these changes there is a considerable increase in extractable collagen in connective tissues (4, 5). In the chick embryo extractable collagen in bones, cartilage, skin, aorta, and tendon appears within an hour following injection, increases rapidly with time of incubation and is dosage-dependent (4, 6–8). Within 72 hours more than half of the total collagen may be extracted in cold 1 M saline whereas in normal embryos less than 1 per cent of the collagen is soluble.

In our efforts to understand how lathyrogenic compounds act to alter collagen solubility we have studied the distribution of isotopically labeled βAPN and its metabolites in the chick embryo, and in particular its relationship to the solubilized collagen. We have attempted to determine whether these agents act through metabolic processes or whether they must be present in the tissues in association or at least in proximity with the affected collagen.

Methods

Fertile eggs of white Leghorn chickens were incubated at 39°C for 14 days at which time cold β-aminopropionitrile fumarate plus 1-C^{14} βAPN·HCl or U-H^3 βAPN·HCl were administered in 0.2 ml of sterile water onto the chorioallantoic membrane through a pinhole in the shell (4).

1-C^{14} βAPN·HCl, specific activity 1.57 mc/mmole, and uniformly labeled H^3 βAPN, specific activity 120 mc/mmole, were obtained from the New England Nuclear Corp., Boston. These compounds, pure by electrophoretic and paper chromatographic criteria, were dissolved in appropriate amounts of sterile distilled water and stored in the frozen state. Materials labeled with C^{14} were measured on stainless steel planchets in a gas-flow counter with a

* This is publication No. 330 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School at the Massachusetts General Hospital, Boston.

‡ Work done during the tenure of a Helen Hay Whitney Fellowship. Present address: Fondation Médicale Reine Elizabeth, Service: Médecine Expérimentale, Bruxelles, Belgium.

background of 1.5 CPM and an efficiency of about 26 per cent. Tritium-labeled compounds were counted in 10 ml of Bray's solution in an automatic liquid scintillation counter with a background rate of 30 CPM and an efficiency of 11 per cent.

After the appropriate periods of incubation following injection of the agent, the yolk, membranes, amniotic fluid, and embryo were separated from each other, weighed, and processed.

Long bones were dissected free of soft tissues and handled separately. These were minced with scissors and extracted three times with 3 volumes of cold 1 M NaCl buffered with phosphate, pH 7.6, Γ/2 0.02. Extraction was carried out in the cold over night with shaking followed by sedimentation at 100,000 *g* for 1 hour. The clear supernatant solutions were then filtered through coarse, medium, and fine sintered glass. All operations were carried out between 0° and 5°C.

Viscosity measurements were made in Ostwald viscometers at 5°C with a flow time for water of about 60 second. Hydroxyproline was measured by the method of Neuman and Logan after hydrolysis in 6 N HCl at 138°C for 3 hours.

Extracted collagen was purified in several ways. (*a*) Extracts were dialyzed extensively against cold 1 M saline, then warmed to 37°C for 3 hours. The heavy opaque gel of collagen was removed by centrifugation, washed in warm saline, and redissolved in cold 1 M NaCl. Thermal precipitation was repeated and the precipitated collagen dissolved in and dialyzed against 0.1 M acetic acid from which it was lyophilized. (*b*) Collagen was precipitated by dialysis against water, redissolved in cold phosphate, pH 7.6, Γ/2 0.4, and reprecipitated by dialysis against 0.01 M Na_2HPO_4. The precipitate was dissolved in 0.1 M acetic acid, dialyzed, and lyophilized. (*c*) Collagen was initially precipitated by the slow addition of an equal volume of cold 5 M NaCl, redissolved in neutral phosphate, and further purified by combinations of methods *b* and *c*.

To obtain free labeled BAPN or its products from the tissues, embryos and extra embryonic structures were extracted in 5 volumes of cold 5 per cent TCA in an ice-cooled high speed blender. The extract was separated in the Spinco preparative ultracentrifuge and clarified by filtration through fine sintered glass. Fractionation of TCA-soluble radioactive substances was accomplished by ion exchange chromatography and by curtain electrophoresis. The extracts were freed of trichloracetic acid by repeated washing with ether, following which they were concentrated in a column of warm air and desalted electrolytically. All stages were checked for losses of radioactivity. Ion exchange separation was accomplished on 5 to 20 ml columns of Dowex 50-X8 in the hydrogen form, eluted with water until neutral followed by 1 M ammonium hydroxide. Certain fractions therefrom were further separated on Dowex-1 formate with water followed by 1 M HCl. Curtain electrophoresis was performed on ether-washed, electrolytically desalted TCA extracts at 11°C in 0.05 M pyridine formate, pH 5.3 at 300 to 400 volts, 18 to 22 ma for 12 to 24 hours. The various radioactive fractions obtained from both the column and curtain were counted, pooled, and further analyzed by paper electrophoresis in potassium acetate buffer, 0.05 to 0.1 M at pH 2.7 and 5.3, at 2000 volts at room temperature. Paper chromatography on the same fractions was also performed in two different solvents; methanol-water-pyridine (4:1:2) and in methyl ethyl ketone-propionic acid-water (174:57:69).

Chromatograms and electrophoresis patterns were monitored in a gas-flow 4π strip counter or by counting small rectangular pieces sequentially cut and placed on stainless steel planchets in a low background gas-flow counter. Ninhydrin was used to stain the cold carriers (βAPN and β-alanine) and diazotized sulfanilic acid was used for cyanoacetic acid and hydracyrlonitrile.

RESULTS

The distribution of C^{14} in embryo homogenates, livers, yolk, extraembryonic membranes, amniotic fluid, and long bones was determined at three different

time intervals after the injection of C^{14} βAPN. Results of three separate experiments are reported in Table I.

About 80 per cent of the total injected radioactivity was accounted for. No radioactivity could be picked up as CO_2 from injected eggs incubated in a desiccator through which air passed into $Ba(OH)_2$ solution over a 24 hour period.

TABLE I

*Distribution of TCA-Soluble Radioactivity in the Chick Embryo at Different Times after C^{14} β-Aminopropionitrile**

Individual experiments	CPM per gm wet weight		
	10 min.	4 hrs.	24 hrs.
Embryos			
1	20,100	16,300	5,110
2	13,400	14,900	21,800
3	—	—	32,500
4	—	—	44,000
5	—	—	48,000
6	—	—	47,000
Livers			
1 + 2	55,100	62,200	57,000
3 + 4	—	—	128,000
5 + 6	—	—	15,000
Extraembryonic structures‡			
1	11,600	19,500	5,700
2	10,100	14,800	5,000
3	—	—	186,000
4	—	—	246,000
5	—	—	124,000
6	—	—	236,000

* Eggs 1 and 2 received 4 μc in 20 mg βAPN fumarate. Eggs 3 to 6 represent two more experiments in which embryos received 20 μc in 20 mg βAPN fumarate.

‡ Includes yolk, membranes and amniotic fluid, and albumin.

Measurement of the distribution of C^{14} between the TCA supernate of homogenized embryos and the residue indicated that approximately 10 per cent is insoluble in trichloroacetic acid.

The radioactive substances extracted from whole embryos and from bone (with cold 1 M NaCl) when fractionated by ion exchange chromatography or by curtain electrophoresis, separated into three major components.

Fig. 1 illustrates a typical column elution pattern of the dialyzable radio-

activity from a 1 M NaCl extract of lathyritic bones. Essentially all the radioactivity (98 per cent) in the bone extracts was dialyzable. Patterns obtained from TCA extracts of whole embryos, livers, and yolk were essentially the same. Fractions 2 and 3, and 13 and 14 were concentrated and examined by paper electrophoresis, at pH 5.3. β-aminopropionitrile, cyanoacetic acid, and a component isoelectric at pH 5.3 were detected (Fig. 2). Paper chromatography confirmed the identification of the two mobile compounds. The third, more neutral fraction behaved like β-alanine on paper chromatography although in

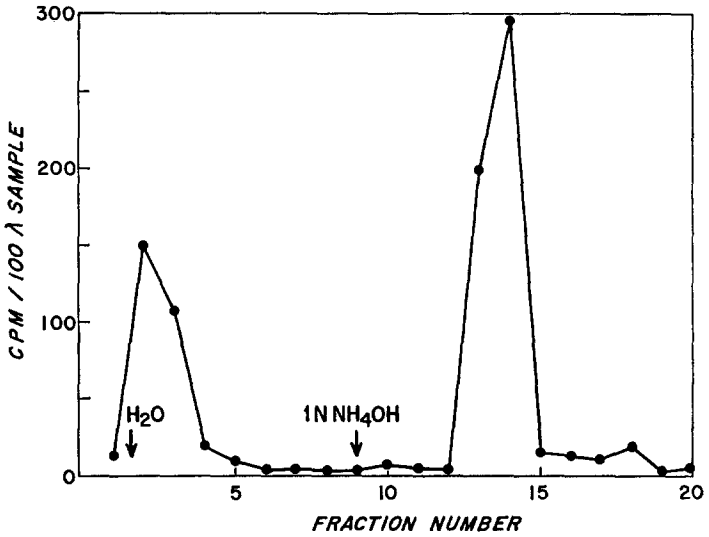

FIG. 1. Elution diagram from Dowex 50 column of dialyzable radioactive compounds in 1 M NaCl extract of lathyritic bones.

several runs it appeared to split into two radioactive components. Curtain electrophoresis of cold embryo extracts at pH 5.3 also revealed three distinct components, the more acid and basic of which were determined to be cyanoacetic acid and β-aminopropionitrile respectively. The more neutral fractions could not be positively characterized. As shown in Table II the distribution of the label is essentially similar in the yolk, in the liver and the carcass 24 hours after administering the agent and in the long bones 48 hours after injection. In 48 hours there appears to be an increased accumulation of the acid-neutral fraction in the whole embryo tissues.

In an effort to determine whether or not β-aminopropionitrile or its breakdown products were bound to the extractable lathyritic collagen eight separate experiments were performed utilizing C^{14}-labeled βAPN and one using βAPN uniformly labeled with tritium. The specific activities of the injected com-

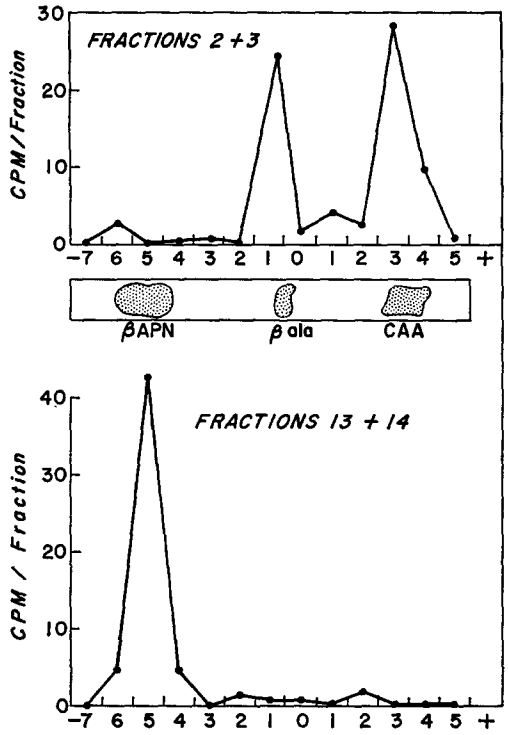

FIG. 2. Top, paper electrophoresis diagram of radioactive compounds eluted from Dowex column in tubes 2 and 3, shown in Fig. 1. Strip shows position of stained reference markers; βAPN = β-aminopropionitrile, βala = β-alanine, CAA = cyanoacetic acid. Bottom, electrophoresis pattern of compounds in tubes 13 and 14, eluted at alkaline pH from Dowex, shown in Fig. 1.

TABLE II

Distribution of C^{14} in the Egg

	Fractions*	
	Acid-neutral	basic
	per cent	*per cent*
Yolk	41	36
Liver	34	58
Carcass‡		
24 hrs.	27	63
48 hrs.	45	45
Bones, 48 hrs.	30	57

* Fraction of total counts recovered from column.

‡ 24 and 48 hours after injection of βAPN.

pounds were high enough so that accurate measurements of radioactivity in the isolated purified collagen at levels several hundred times below a hypothetical equimolar complex of βAPN and collagen could be made. The results of these experiments are reported in Table III. Preparations 5 to 9 represent the most highly purified collagens. Preparation 5 was dialyzed against large volumes of 0.1 M cold βAPN in saline, then water prior to lyophilization. The largest amount of radioactivity found represented 1 mole of C^{14}-labeled compound per 5 moles of collagen in the first and most impure preparation. Less

TABLE III

*Association between β-Aminopropionitrile and Purified Extracted Lathyritic Collagen**

	Measured specific activity		
Compound	βAPN injected	Collagen extracted	Molar ratio βAPN: collagen
	CPM/μmole	CPM/μmole	
1. 1-C^{14}-βAPN	6.4×10^4	12×10^3	1:5
2. 1-C^{14}-βAPN	6.4×10^4	1.5×10^3	1:42
3. 1-C^{14}-βAPN	6.4×10^4	3.3×10^3	1:20
4. 1-C^{14}-βAPN	6.4×10^4	4.5×10^3	1:14
5. 1-C^{14}-βAPN	6.4×10^4	0.6×10^3	1:100
6. 1-C^{14}-βAPN	6.4×10^4	0.45×10^3	1:140
7. 1-C^{14}-βAPN	6.4×10^4	0	0
8. 1-C^{14}-βAPN	1.6×10^4	0.3×10^3	1:53
9. U-H^3-βAPN	10.6×10^5	6.0×10^3	1:180

* Nine separate experiments.

than 1 mole of labeled compound per 100 moles of collagen were found in three of the C^{14} experiments and in the tritium-labeled preparation.

A complete column chromatographic amino acid analysis of purified extracted lathyritic chick embryo bone failed to demonstrate the presence of β-alanine, an expected hydrolysis product of βAPN.[1] No deviation from the normal amino acid distribution was found.

The possibility that lathyrogens might cause the solubilization of collagen without firmly bonding to the protein was explored by dialyzing minced lathyritic bone against saline to remove the agent, then comparing collagen solubility with that of suitable controls.

Twenty embryos were each injected with 20 mg of cold βAPN plus 5 μc of C^{14}-labeled compound at 14 days of incubation. Long bones were isolated 48 hours later, pooled, minced,

[1] Column chromatographic analyses on normal and lathyritic-extractable chick embryo and guinea pig collagens were performed for us by Dr. Karl Piez at the National Institute for Dental Research.

and separated into equal aliquots. These were suspended in several milliliters of water in dialysis bags. Sample I was suspended in a moist flask as a mock dialysis; sample II dialyzed against 0.1 M βAPN in saline; sample III was dialyzed against 0.1 M saline. The experiment was performed at 5°C and 37°C. After 48 hours during which time the dialyzing fluids were changed repeatedly, the contents of the dialysis bags were suspended in two volumes of cold

TABLE IV

*Extractability of Collagen from Bones after Removal of βAPN by Dialysis**

Treatment	Hydroxyproline in μg/ml extract		
	5°C	37°C	
		I	II
	μg/ml	μg/ml	μg/ml
I. Mock dialysis	360	265	520
			640
II. Dialysis *vs.* βAPN	360	360	410
			450
III. Dialysis *vs.* NaCl	365	275	210
			340
IV. Normal bones‡	10	15	—

* Two separate experiments, I and II were performed at 37°C; II was done in duplicate.

‡ The values for extractable hydroxyproline for normal bones were obtained from a separate experiment.

TABLE V

Effect of Temperature of Incubation on Collagen Extractability

Temperature of incubation for 24 hrs.*	Average weight	η_{rel} bone extracts
°C	gm	
38	10.2 ± 1.1	27.6
31	9.7 ± 1.1	20.0
25	8.9 ± 1.1	11.9

* Embryos incubated for 14 days at 38°C before experimental period.

‡ Long bones of 18 embryos in each group pooled and extracted.

1 M saline and extracted on the shaker at 4°C for 24 hours. The separated extracts were analyzed for radioactivity to determine the extent of removal of βAPN in the dialysis step. Hydroxyproline content was used as a measure of the amount of extractable collagen.

Essentially all the βAPN or its breakdown products were removed from the tissues by dialysis as there was no measurable radioactivity in the subsequent salt extracts. At 37°C the amounts of extractable collagen were lower in the samples dialyzed against saline (Table IV). However the level of extract-

ability was such as to suggest that the presence of βAPN, or its metabolites in the environment of collagen was not essential for solubilization.

The obvious direct experiment was also performed, in which normal chick bones were incubated for 1 to 5 days in the presence of varying concentrations of βAPN in Tyrode's solution, in tissue culture media, or in tissue fluids from lathyritic embryos. There was no increase in collagen solubility over that of controls. Martin (9) has reported an increase of extractable collagen in βAPN-treated bone cultures after 12 days of incubation.

In an effort to determine whether or not the solubilization mechanism depended upon metabolic processes, the effect of varying the temperature of incubation of the eggs upon the degree of solubilization of collagen in lathyritic embryos was examined. Three groups of 20 eggs were injected at 14 days with 20 mg of βAPN each and then incubated for 24 hours more at three different temperatures, 27°, 32°, and 38°C. Table V reveals that the amount of extractable bone collagen was proportional to the temperature of incubation as was the growth rate. In another related group of experiments lathyritic embryos were killed with KCN or by freezing 6 hours after administration of βAPN. The production of extractable collagen stopped at that point even though incubation at 37°C continued for another 16 hours.

DISCUSSION

The chick embryo, being a closed system from which there is little loss by way of excretion, provides a better test animal for this type of experiment than does the rat since all the metabolic products are retained. It would appear that β-aminopropionitrile is distributed throughout the egg within 10 minutes after administration. The greatest bulk of this material is soluble in TCA and in the case of bone is almost completely dialyzable. Even after 48 hours more than 40 per cent of the radioactivity can be accounted for by unaltered β-aminopropionitrile. The next largest radioactive component is the known metabolic product, cyanoacetic acid (10) which itself has no lathyrogenic activity. Nothing certain can be said at this time about the identity of the remaining 20 per cent of the radioactive material although it would appear likely that two of these compounds are β-alanine and hydracrylonitrile (cyanoethanol). Neither of them are lathyrogenic in the chick embryo. No attempt has been made to identify the radioactive components in the TCA-insoluble fraction. In an earlier study of the distribution of C^{14}-labeled aminoacetonitrile (11) in the rat it was observed that some of the amino acids in protein had become labeled.

It has been proposed by Levene (12) that lathyrogens act to solubilize collagen by binding onto aldehyde groups within the collagen molecule thereby blocking intermolecular cross-linking. If this were the case one should find at least one molecule of βAPN for two molecules of collagen. In this study we

have observed less than 1 mole of βAPN per 100 moles of collagen, utilizing both C^{14}-labeled and uniformly tritium-labeled compounds. The use of the uniformly labeled compound eliminates the likelihood of a collagen-bound derivative of βAPN. Stegemann (13) has recently reported the presence of β-alanine, the hydrolysis product of βAPN in lathyritic collagen hydrolyzates. No β-alanine was found in our purified preparations although it may be present in the free dialyzable form in the tissues. Thus it is unlikely that the lathyrogenic agents operate by direct combination with collagen. In addition, we have shown that the collagen in lathyritic chick embryo bone is still extractable after complete removal of βAPN by extensive dialysis. It is therefore also unlikely that the presence of the agent in the vicinity of the collagen fibrils is essential for solubilization. We are left with the likelihood that in some manner the collagen structure has been altered by a metabolically related process so that firm intermolecular bonding is not present. An alternative explanation might involve an association with another unidentified product, unrelated to the lathyrogen, which interferes with firm cross-linking.

The fact that extractability is directly related to temperature of incubation of the eggs and that the progressive increase in extractability ceases abruptly with the death of the animal suggests that metabolic processes are involved. The observations (14) that purified reconstituted lathyritic guinea pig skin collagen remains reversibly soluble after more than a week of incubation at body temperature, in contrast with the normal, further suggests a subtle structural alteration. Whether or not the observed intramolecular change in ratio of α to β components in lathyritic collagen (15, 16) is directly related to the alteration in solubility is still an open question. At this time it is not certain whether the mechanism involves formation of an abnormal collagen (4) or the alteration of previously deposited, normal, insoluble fibrils (8).

The authors wish to thank Miss Barbara McKee for her invaluable assistance.

SUMMARY

1. C^{14}-labeled β-aminopropionitrile distributed throughout the egg contents within 10 minutes postinjection. By ion exchange chromatography and electrophoretic analysis three major components of the extractable dialyzable radioactive material could be demonstrated, representing at least 80 per cent of the total. The acidic and basic components were identified as β-aminopropionitrile and cyanoacetic acid, while the fraction isoelectric at pH 5.3, consisting of two components, remained unidentified.

2. Less than 1 molecule of βAPN per 100 molecules of protein was present in the highly purified extractable lathyritic bone collagen indicating that binding of the lathyrogen is not a factor in collagen extractability.

3. The proximity of βAPN to collagen in bone is not essential to its extractability.

4. The effect of incubation temperature of the embryo on collagen extractability suggests the involvement of a metabolic process in this phenomenon.

BIBLIOGRAPHY

1. Geiger, B. J., Steenbock, H., and Parsons, H. T., Lathyrism in the rat, *J. Nutrition*, 1933, **6**, 427.
2. Ponseti, I. V., and Shepard, R. S., Lesions of the sekeleton and other mesodermal tissues in rats fed sweet pea (*Lathyrus odoratus*) seeds, *Am. J. Path.*, 1952, **28**, 1059.
3. Selye, H., Lathyrism, *Rev. canad. biol.*, 1957, **16**, 1.
4. Levene, C. I., and Gross, J., Alterations in state of molecular aggregation of collagen induced in chick embryos by β-aminopropionitrile (lathyrus factor), *J. Exp. Med.*, 1959, **110**, 771.
5. Mikkonen, L., Tuominen, T., and Kulonen, E., Collagen fractions in lathyritic rats, *Biochem. Pharmacol.* 1960, **3**, 181.
6. Gross, J., Levene, C. I., and Orloff, S., Fragility and extractable collagen in the lathyritic chick embryo. An assay for lathyrogenic agents, *Proc. Soc. Exp. Biol. and Med.*, 1960, **105**, 148.
7. Tanzer, M. L., and Gross, J., data in preparation.
8. Smiley, J. D., Yeager, H., and Ziff, M., Collagen metabolism in osteolathyrism in chick embryos: Site of action of β-aminopropionitrile, *J. Exp. Med.*, 1962, **116**, 45.
9. Martin, G. R., The action of aminoacetonitrile on bone in tissue culture, *Biochim. et Biophysica Acta*, 1963, **69**, 568.
10. Lipton, S. H., Lalich, J. J., and Strong, F. M., Identification of cyanoacetic acid as a metabolite of β-aminopropionitrile (βAPN) and other nitriles, *J. Am. Chem. Soc.*, 1958, **80**, 2022.
11. Ponseti, I. V., Wawzonek, S., Franklin, W. E., Winnick, R. E., and Winnick, T., Distribution of C^{14}-labeled aminoacetonitrile in tissues of rats, metabolism and mode of elimination, *Proc. Soc. Exp. Biol. and Med.*, 1956, **93**, 515.
12. Levene, C. I., Studies on the mode of action of lathyrogenic compounds, *J. Exp. Med.*, 1962, **116**, 119.
13. Stalder, K., and Stegemann, J., Incorporation of β-aminopropionitrile into the collagen molecule in lathyrism in the rat, *Naturwissenschaften*, 1962, **49**, 398.
14. Gross, J., An intermolecular defect of collagen in experimental lathyrism, *Biochim. et Biophysica Acta*, 1963, **71**, 249.
15. Piez, K. A., Lewis, M. S., Martin, G. R., and Gross, J., Subunits of the collagen molecule, *Biochim. et Biophysica Acta*, 1961, **53**, 596.
16. Martin, G. R., Gross, J., Piez, K. A., and Lewis, M. S., On the intramolecular cross-linking of collagen in lathyritic rats, *Biochim. et Biophysica Acta*, 1961, **53**, 599; Martin, G. R., Piez, K. A., and Lewis, M. S., The incorporation of [^{14}C] glycine into the subunits of collagens from normal and lathyritic animals, *Biochim. et Biophysica Acta*, 1963, **69**, 472.