COLLAGEN METABOLISM IN OSTEOLATHYRISM IN CHICK EMBRYOS: SITE OF ACTION OF β-AMINOPROPIONITRILE*, ‡

By J. DONALD SMILEY,§ M.D., HENRY YEAGER,‖ M.D., AND MORRIS ZIFF, M.D.

(From the Department of Internal Medicine, Rheumatic Diseases Unit, The University of Texas Southwestern Medical School, Dallas)

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When administered to intact animals, lathyritic agents alter the state of molecular aggregation of collagen, increasing the fraction which may be extracted by dilute acid or neutral salt solutions (2, 3). This alteration results in profound pathological changes in the treated animal (4), and provides a useful experimental tool for the study of reactions responsible for collagen fiber formation.

Gross and Levene (2, 5) have suggested that lathyritic agents produce their effects by bringing about the solution of mature collagen fibers. They showed a decline in the concentration of collagen in the non-extractable residue of lathyritic chick embryo skin, aorta, and bone, while control animals showed a continuous rise in non-extractable collagen concentration during the same period. Follis and Tousimis, on the other hand, (6) have proposed that lathyritic agents prevent the formation of fibrils from normally synthesized tropocollagen on the basis of observation of a decrease in the number of collagen fibers in lathyritic rat cartilage while the hydroxyproline content of this tissue remained unchanged.

These varying interpretations have prompted a re-examination of the problem, utilizing incorporation of proline-C^{14} into the extractable and non-extractable fractions of collagen in chick embryos rendered lathyritic by means of β-aminopropionitrile[1] (BAPN). The results support the conclusion that lathyritic agents inhibit the formation of mature fibers in the presence of otherwise normal collagen synthesis.

Materials and Methods

The methods used were essentially those of Levene and Gross (2) with variations as indicated below.

* A preliminary report of this work has been submitted (1).

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§ Special Research Fellow, National Institute of Arthritis and Metabolic Diseases.

‖ United States Public Health Service Trainee in Arthritis.

[1] β-aminopropionitrile (BAPN) was obtained from Abbott Laboratories, North Chicago.

Injection of Embryos.—In the first experiment, fertilized 14-day-old Rhode Island Red eggs were injected in the air sac with 0.1 ml of a solution containing 7.5 mg of BAPN (neutralized immediately before use with N NaOH) and 0.1 ml of an isotonic saline solution containing 1 μc of L-proline-C^{14} (Nuclear-Chicago, 5 mc/mmole). By placing a second hole in the shell over the air space, leakage losses were minimized, and rapid, uniform absorption of the drug and C^{14}-label were achieved (7). Control eggs were injected with 1 μc of L-proline-C^{14} and 0.1 ml of isotonic saline. Eggs were incubated at 40°C.

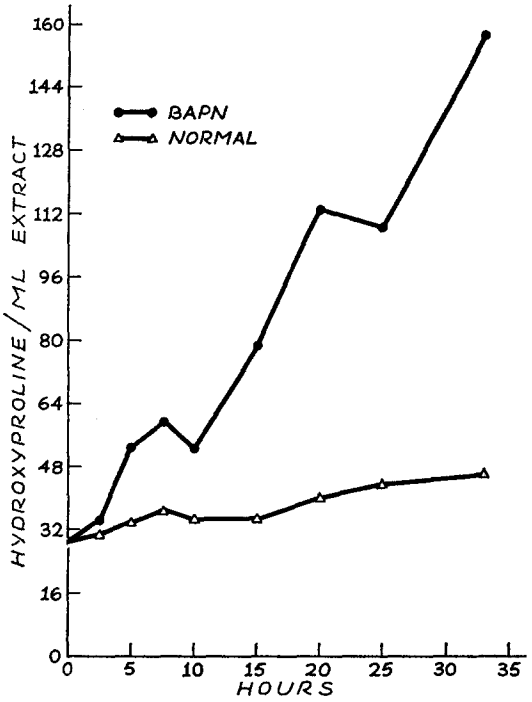

FIG. 1. Increase in soluble collagen following BAPN administered at 0 time.

In the second experiment, fertilized eggs of the same variety were injected in the air sac on day 13 of incubation with 1 μc of L-proline-C^{14} and 24 hours later with 7.5 mg of BAPN in 0.1 ml. Subsequent procedure was identical with that in the first experiment.

Separation of Collagen Fractions.—Groups of five or six embryos in experimental and in control groups were sacrificed at indicated time intervals, and the head, viscera, and feathers removed. The remainder was minced finely with scissors at 0°C, weighed, and aliquots taken for dry weight determinations. 2 ml of N NaCl in 0.02 M sodium phosphate buffer, pH 7.6, was then added per gram of minced tissue and the suspension extracted with continuous shaking for 24 hours at 0°C. Foam was eliminated by centrifuging briefly at 25,000 *G* in a Servall model SS-1 centrifuge and then at 100,000 *G* for 45 minutes at 0°C in a Spinco model L preparative ultracentrifuge. The supernatant was poured off as a clear, red, viscous solution referred to hereafter as the soluble fraction. The pellets remaining were pooled and wet weight determined. The pellets were then homogenized in a Lourdes mastermixer with 100 ml of water. This homogenate will be referred to as the residue fraction.

Analytical Methods.—Aliquots of both the soluble fraction and the homogenate of the residue fraction were adjusted to 6 N HCl by addition of concentrated acid, sealed in glass tubes, and hydrolyzed in an autoclave at 124°C for 15 hours. The tubes were then opened and their contents evaporated to dryness *in vacuo* over NaOH pellets. For determination of hydroxyproline, the dry residue was taken up in water and analyzed by the method of Prockop and Udenfriend (8) with minor modifications (9).

For measurement of specific radioactivity, an aliquot of each fraction containing about 2

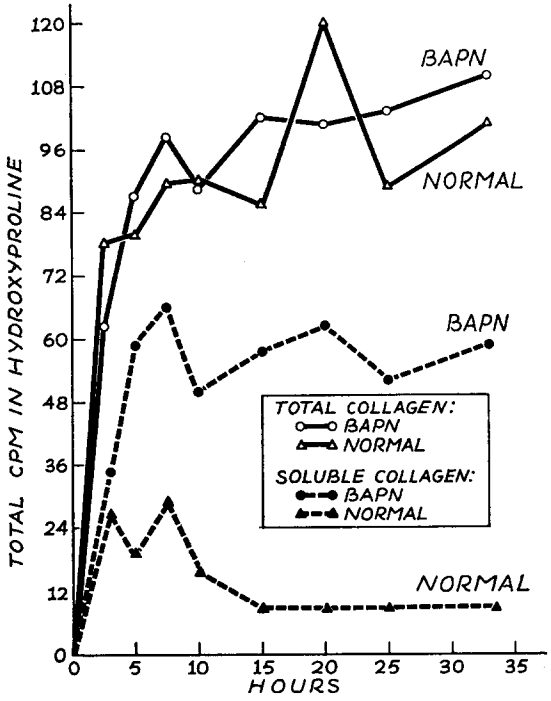

FIG. 2. Distribution of total radioactivity in counts per minute (CPM) following administration of BAPN and proline-C^{14} at 0 time.

μ moles of hydroxyproline was hydrolyzed and evaporated to dryness as above. Each sample was dissolved in 95 per cent ethanol, cleared of insoluble material by centrifugation, and the supernatant streaked on sheets of Whatman No. 1 filter paper. These were chromatographed in phenol:water (90:10) at 40°C for 40 hours (10). Hydrox, proline was located on a narrow center strip cut from each paper by staining with a ninhydrin-isatin dip (11).[2] The hydroxyproline was then eluted and oxidized to pyrrole, which was used for determination of specific radioactivity (12). A Packard tri-carb liquid scintillation spectrometer and standard C^{14} counting techniques were employed.

It is assumed in this paper that hydroxyproline is a measure of collagen since its tissue distribution is limited primarily to this protein (13).

[2] Tributylamine was substituted for triethylamine since it gave more intense color development with hydroxyproline.

RESULTS

Action of BAPN on Collagen Synthesis and Fiber Formation (Experiment 1).—BAPN in the dosage given produced severe lathyrism in the chick embryos. This was reflected by detectable increases in soluble collagen above that of controls within 2.5 hours (Fig. 1). There was no interference with over-all collagen synthesis, however. This is best illustrated by comparing the total incorporation of proline-C^{14} into hydroxyproline-C^{14} in the BAPN and control groups (Fig. 2). Values are averages of two or more determinations on the pooled tissue of six animals.

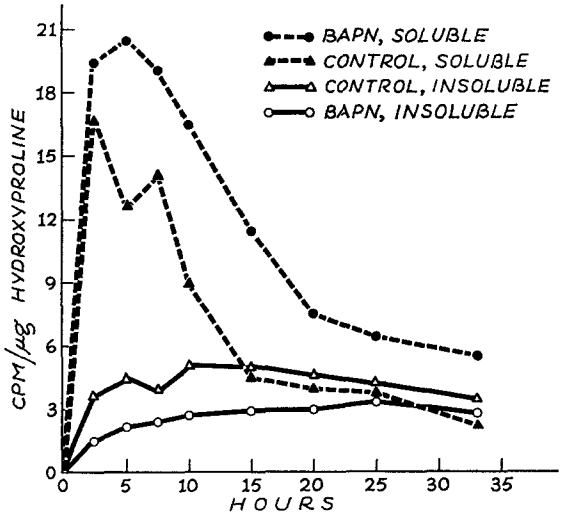

FIG. 3. Specific radioactivity following administration of BAPN and proline-C^{14} at 0 time.

When hydroxyproline was separated from hydrolysates of soluble and residue fractions and specific radioactivity determined, three differences were observed between the BAPN-treated and control animals. Although the treated animals incorporated proline into the hydroxyproline of the soluble fraction as rapidly as the controls initially (Fig. 3), the maximum specific activity of the soluble fraction attained in the BAPN-treated group was greater than in the control group. In addition, the decline of the specific activity of the soluble collagen fraction in the BAPN-treated group was slower than in the untreated embryos. Finally, there was a decreased rate of incorporation of C^{14} into the collagen of the residue fraction of the treated group.

These results appeared to be most compatible with the presence in lathyritic animals of a partial blockade of new fiber formation, as will be discussed below.

Administration of BAPN following Proline-C^{14} (Experiment 2).—To investi-

gate further the effect of BAPN on the insoluble residue collagen, it appeared desirable to administer this agent at a time when the insoluble fraction was more highly labeled than the extractable collagen. This labeling pattern was achieved by administration of proline-C^{14} 24 hours before injection of BAPN. (Fig. 4). This pattern had been previously observed in the preceding experiment in the control group 24 hours after administration of proline-C^{14} (Fig. 3).

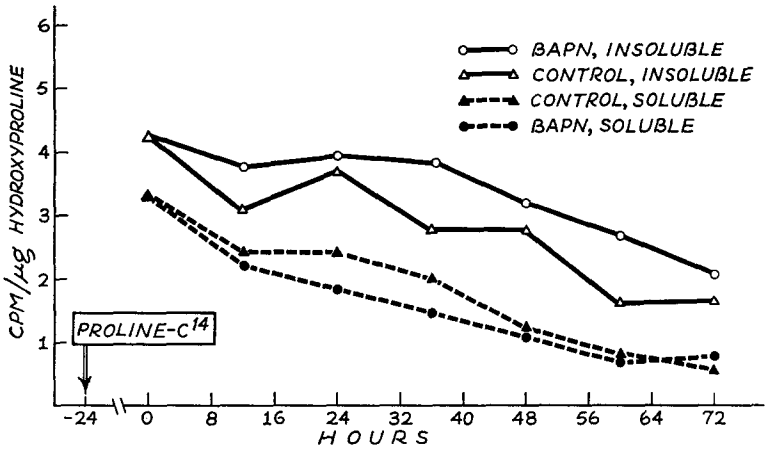

FIG. 4. Specific radioactivity following administration of BAPN at 0 time. Proline-C^{14} was injected 24 hours earlier.

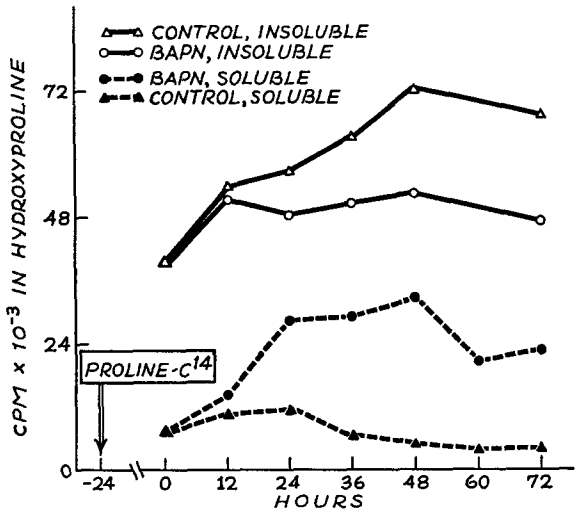

FIG. 5. Total radioactivity of hydroxyproline in collagen fractions. BAPN was administered at 0 time and proline-C^{14} 24 hours earlier.

In spite of the greater specific activity of the residue collagen prior to injection of BAPN, there was no increase in the observed specific radioactivity of the newly formed, soluble collagen following the administration of this compound. The specific activity of the soluble collagen, in fact, remained below that of controls. These results seem best explained by the maintenance of the integrity of the mature collagen fibers following BAPN.

Total Collagen and Total Radioactivity after Administration of BAPN.—The distribution of total radioactivity in the hydroxyproline of the soluble and insoluble fractions of experiment 2 were calculated from the specific activity and hydroxyproline content of these fractions (Fig. 5). Total radioactivity of the hydroxyproline of the residue collagen increased only slightly following

TABLE I

Hydroxyproline of Total and Residue Collagen During Course of Experiment 2 (mg/gm dry weight original tissue)

Normal				BAPN-treated	
Day 14		Day 17		Day 17	
Total	Residue	Total	Residue	Total	Residue
2.68	2.12	6.61	5.86	7.06	2.90

Dry weight determinations varied between 12 and 14 per cent of wet weight.

administration of BAPN, whereas, in control animals, the C^{14}-label continued to be incorporated into the hydroxyproline of the insoluble collagen fibers. In addition, at the time of maximum rate of increase of radioactivity within the soluble collagen fraction, there was no decrease in radioactivity in the insoluble fraction of the BAPN-treated embryos. These findings again suggest maintenance of the integrity of the residue collagen fibers during the period of maximum drug action.

Consistent with a block in the formation of mature fibers, the collagen concentration of the residue fraction increased only slightly in the BAPN-treated embryos in contrast to the marked increase in this fraction in the untreated controls (Table I).

DISCUSSION

Orekhovitch and his coworkers (14) and, more recently, several other groups (15–17) have suggested varying degrees of molecular cross-linking to account for the observed heterogeneity of soluble collagen, whether derived by neutral salt or dilute acid extraction procedures. Martin and coworkers (18) have recently shown that the extractable collagen of BAPN-treated animals lacks the ability to form chain pairs (β-collagen) during *in vitro* incubation at 38°C

and consists predominantly of unpaired chains (α-collagen) when chromatographed on carboxymethyl cellulose. This is in contrast to 1 M NaCl extractable collagen of normal animals which contains both α- and β-collagen forms (19, 20). Jackson (20) has shown that the α-collagen fraction has the highest specific radioactivity immediately following glycine-C^{14} administration and has suggested that 0.14 M NaCl extracts of microsomes of fibroblasts synthesizing new collagen in carrageenin granulomas contain only the α-collagen form.

These observations provide a framework (Fig. 6) upon which to explain the data obtained in the experiments reported here. According to this view, single-chain, α-collagen units undergo progressive cross-linkage through ester or

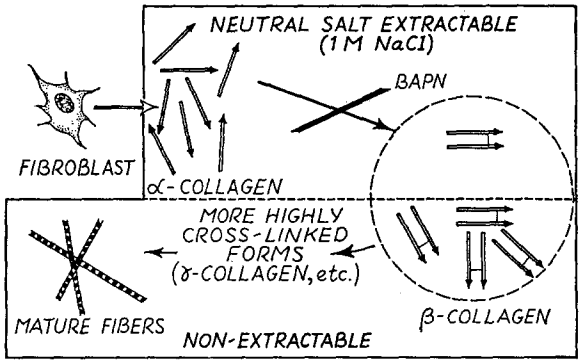

FIG. 6. Proposed site of action of BAPN on collagen metabolism in osteolathyrism.

other bond formation (21) to form mature, non-extractable collagen. The data obtained in the present investigation suggest that lathyritic agents such as BAPN block cross-linking, covalent bond formation, possibly by interference with an as yet clandestine enzyme system.

Following administration of proline-C^{14} one would expect the highest specific activity to appear first in the newly synthesized α-collagen fraction. In normal embryos, the radioactivity would quickly pass into the β-collagen fraction and finally into the γ-collagen and mature collagen fibers. In the experimental group, however, because of the BAPN-induced block, the α-collagen fraction would increase rapidly in specific radioactivity as well as in size during the initial phase of the experiment.

Since β-collagen and more highly cross-linked forms are progressively less extractable (20), the amount of β-collagen appearing in the 1 M NaCl soluble extract, though significant in comparison with the amount of α-collagen, may represent only a small portion of a large pool of relatively less radioactive material. It is not surprising, therefore, that the specific activity of the 1 M extract in the normal animals, containing both α- and presumably significant

amounts of less radioactive β-collagen, reached a somewhat lower maximum than that of the lathyritic animals (Fig. 3). The absence of a BAPN block in the normal animals would also explain the more rapid decline of the specific radioactivity of the extractable collagen of the control animals.

In the case of the residue collagen, it would be expected that the specific activity of this fraction in the lathyritic embryos would be less than that of their corresponding controls, as was observed. No block in synthesis of collagen was produced by BAPN, as shown by the similarity in the total amount of radioactive proline converted to hydroxyproline during the time period of the experiment in control and treated embryos (Fig. 2).

In contrast to the findings of Levene and Gross (2) in skin, aorta, and bone, where residue collagen concentration was found to decline sharply from initial levels after administration of BAPN, the present experiments showed (Table I) a leveling off of residue collagen concentration but no actual decline. Certain aspects of the present experiments might account for the differences observed in the residue fraction. The experiments here reported were performed on the almost intact embryo rather than on isolated skin, bone, or aorta. Also, the route of BAPN administration, variety of chick embryo, and temperature of incubation were different (2).

There was no evidence that breakdown of mature fibers occurred other than the normal amount of catabolism present in young animals. In the experiments in which proline-C^{14} was administered prior to the BAPN, the specific activity of the insoluble collagen was greater than that of the soluble at the time of injection of BAPN. If insoluble fibers had been rendered soluble, one would have expected the soluble fraction to have increased in specific radioactivity relative to that of the control animals (Fig. 4) and the total counts in the hydroxyproline of the insoluble fraction to have fallen (Fig. 5). Since this was not the case, it seems more likely that the increase in soluble collagen produced by the action of BAPN represented new synthesis of α-collagen with interruption of further conversion to the fibrous form.

Although production of lathyrism in adult animals is well established (4, 5), the changes induced in adults are more restricted than in growing animals, particularly to the more metabolically active tissues (5, 22). Neuberger and Slack (23) have shown that while there is only slight incorporation of glycine-C^{14} into the collagen of most tissues of the adult rat, fairly rapid incorporation occurs into bone. They suggested a continual slow replacement even in adult animals, particularly of bone collagen. This could account for the production of lathyrism with primarily skeletal changes in adult animals. One might also wonder whether a block in cross-linkage, and thus in the mature fiber formation necessary for minor repair, could lead to an abnormal stimulation of new collagen synthesis and to the observed increases in extractable collagen in adult animals treated with BAPN.

In the young, growing animal, Neuberger and Slack (23) found a narrow range of variation of specific radioactivity for the collagen from various tissues. This finding is important for the interpretation of the present observations, since it would tend to indicate that the collagen derived from the almost intact embryo, as employed in these experiments, would have approximately the specific radioactivity as the collagen of the individual tissues at any given time.

SUMMARY

Chick embryos were sacrificed at intervals after simultaneous injection of BAPN and proline-C^{14}, the collagen separated into neutral salt–extractable and residue fractions, and total hydroxyproline and hydroxyproline specific radioactivity determined in each fraction. Extractable collagen, measured as hydroxyproline, increased markedly and had a higher specific activity in BAPN-treated embryos than in corresponding controls. Hydroxyproline of the residue collagen in the treated animals, however, had a lower specific activity.

When proline-C^{14} was injected 24 hours prior to BAPN, the specific radioactivity of the soluble collagen of treated embryos was similar to that of controls, in spite of the fact that the specific activity of the residue fraction was higher than that of the soluble fraction at the time of BAPN administration.

These results suggest that the increased amount of soluble collagen in lathyrism induced by administration of BAPN does not arise from the collagen insoluble prior to administration of the drug, but rather that BAPN acts by blocking the *formation* of mature collagen fibers, perhaps by preventing the formation of cross-linkages between α-collagen chains.

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