block enzyme activity and crosslink formation.

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A Molecular Defect in Lathyritic Collagen.* (31764)

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Experimental lathyrism is a disease of connective tissues, characterized by the occurrence of hernia, aneurysm, and skeletal deformity(1). Underlying the disease is a dramatic increase in the amount of soluble collagen present in the tissues, apparently resulting from a decreased degree of covalent, interchain crosslinking(2,3). A similar defect appears to occur in elastin.

One of the basic problems concerning this disease has been the question of whether lathyrogens inhibit new crosslink formation or cause a rupture of previously-formed crosslinks. The answer to this question is important since it will determine how and where one looks for the molecular defect. Tracer kinetic analysis has been used but the results have led to conflicting conclusions(4,5). This problem was partially resolved by determining the relative rates of accumulation of soluble, insoluble, and total collagen in whole normal and lathyritic chick embryos(6). The data showed that the most likely source of lathyritic collagen is newly aggregated fibrils synthesized after lathyrogen administration. Therefore, lathyrogens seem to exert their effect on connective tissues by causing inhibition of new covalent crosslink formation rather than by rupturing previously-formed crosslinks.

There appear to be two likely mechanisms by which lathyrogens may inhibit the formation of new covalent crosslinks. (a) Lathyrogens may induce structural defects in the crosslinking portion of collagen and elastin molecules, or (b) they may affect a compo-

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nent other than collagen and elastin which is necessary for crosslink formation.

Experiments were designed to obtain information as to whether or not the excess soluble collagen which accumulates in the tissues of lathyritic animals is structurally normal. Since at least one type of covalent, interchain crosslink in collagen appears to derive from amino acids with charged side-chain groups(7), alterations in the crosslinking portion of the molecule would be expected to produce concomitant alterations in the electrophoretic and chromatographic properties of the component alpha-chains and in some of their derived peptides. Therefore, the techniques selected to demonstrate the suspected intrinsic defect in lathyritic collagen were starch-gel electrophoresis and peptide fingerprinting. Since recently reported data suggest that crosslink formation may occur subseqent to aldehyde production(7-9), the various salt and acid soluble collagens were examined for aldehyde content.

Experimental procedures. Weanling Sprague-Dawley rats were made lathyritic by placing 0.4% beta aminopropionitrile fumarate† (BAPN) in their food for 20 days. Collagen was obtained from the skins of normal control, pair-fed control, and lathyritic rats by extraction in 10 volumes of 1 M formic acid at 4°C for 24 hours. This procedure removes all of the excess soluble collagen in a single extraction. To preclude the loss of altered chains, subunits, or chain fragments, the extracts were centrifuged at 25,000 × *g* for 1 hour, lyophilized, and used without dialysis or further purification.

Starch gels were prepared using 70 g of starch (Starch-Hydrolyzed, Connaught Research Laboratories, Toronto, Canada) in 500 ml of buffer containing 6 M urea as previously described by Benditt and Eriksen(10). Buffers used and the approximate beginning milliamperages were 0.01 M sodium formate pH 2.4 to 3.5, ma 62 to 18; 0.02 M sodium acetate pH 4.1 to 6.4, ma 18 to 25; 0.025 M tris HCl pH 7.0 to 8.0, ma 23 to 80; 0.01 M sodium borate pH 8.0 to 9.0, ma 7 to 10; 0.01 M sodium glycinate pH 9.7 to 11.7, ma 11 to 55. Bridge buffers were 10 times more concentrated than gel buffers.

The electrophoresis was carried out for 24 hours at room temperature in a gel sheet 30 cm × 12.4 cm × 0.6 cm using a potential of 120 volts between the terminals. Protein concentration was 20 mg/ml. Gels were sliced and stained with 0.5% nigrosin in ethanol: acetic acid:water (15:3:15) for 5 minutes then decolorized with the same solvent. The procedure used to demonstrate the more slowly migrating collagen species (Fig. 2) was identical except that gels were run at 4°C for 48 hours. The various bands on the patterns were identified by running concurrent samples of purified alpha- and beta-collagens obtained by chromatography on polyacrylamide P-300 columns and shown to be alpha- and beta-collagens by ultracentrifugation.

Enzymatic digests for peptide fingerprinting were prepared by incubating 0.5% (W/V) collagen in 0.1 M pyridine acetate pH 5.3 with 1:10 (W/W) concentration of crystalline chymotrypsin (Worthington Chromatographically Purified), at 43°C for 2 hours while stirring. Electrophoresis of digests prepared under various conditions of time, pH, and temperature showed that the conditions used digested all of the collagen and produced discrete peptides. The digests were lyophilized and fingerprints prepared on #3 Whatman paper from a sample of 10 μl of a 10% solution (W/V). The electrophoretic phase was run at 1,000 volts and 33 ma in 0.018 M acetic acid with s-collidine at pH 5.9. Descending chromatographic separation was in butanol:acetic acid:water (12:3:4). Fingerprints were dried, sprayed with ninhydrin and heated to 80°C for color development.

Collagen samples used for aldehyde determination were sequentially extracted from skin with 0.28 M sodium chloride, 1.0 M sodium chloride and 1 M formic acid. These extracts were purified by the method of Piez, *et al*(11), and frozen in 0.1 M acetic acid until used. Samples were thawed, dialyzed against two changes of 0.1 M glycine buffer pH 4.0 and centrifuged at 105,000 × *g* for 1 hour. Aliquots were hydrolyzed for hydroxyproline determination by the method of

† Beta aminopropionitrile fumarate was the generous gift of the Abbott Co., North Chicago, Ill.

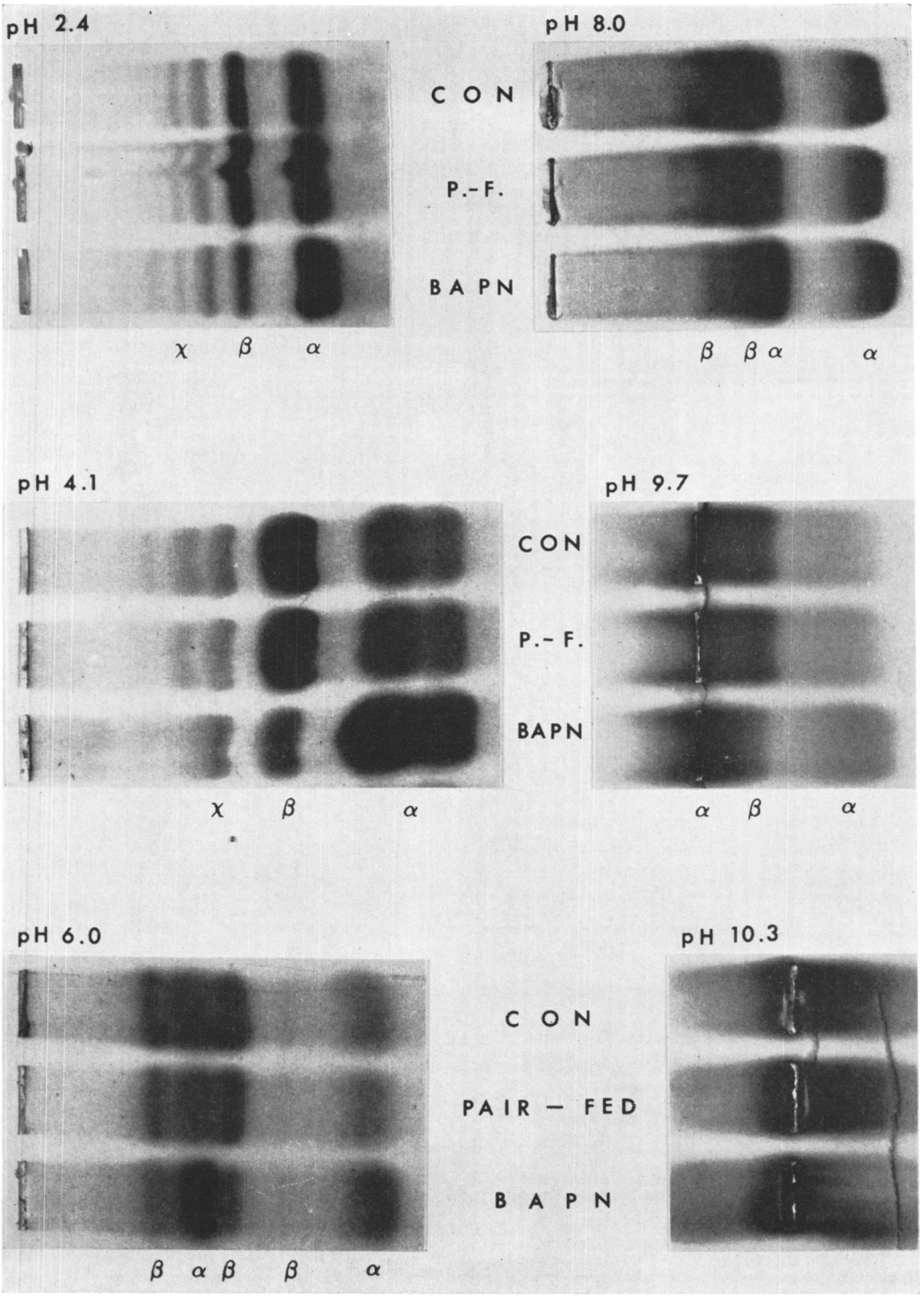

FIG. 1. Representative starch gel electrophoretic patterns of formic acid extracted skin collagen from normal control, pair-fed control, and lathyrogen-treated rats.

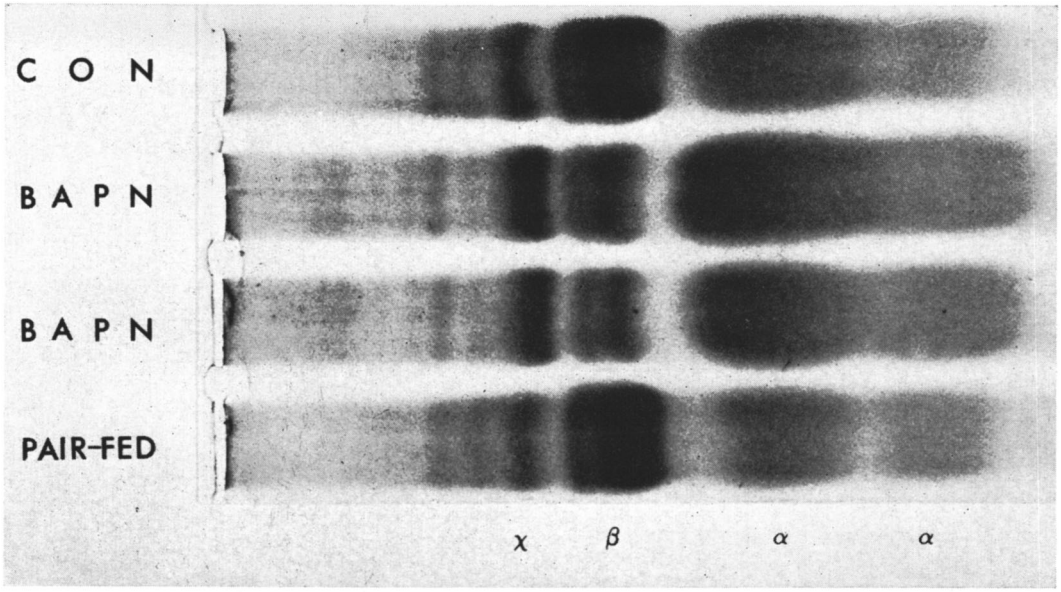

FIG. 2. Starch gel pattern of formic acid extracted skin collagen from normal control, pair-fed control, and lathyrogen-treated rats obtained at pH 4.3 under conditions described under experimental procedures.

Bergman and Loxley(12) modified as previously described(6). The hydroxyproline values were used to determine collagen concentration.

Aldehyde content of the collagen was determined by the azine method of Paz *et al* (13) and quantitated as described by Miller and Fullmer(14) by comparing absorption at 312 mμ with that of a 1:1 mixture of glyceraldehyde and crotonaldehyde carried through the same procedure. Tests containing collagen or standard mixtures of aldehydes were incubated at 40°C and scanned periodically in the spectrophotometer until absorption at 312 mμ became maximal.

Results. Representative starch-gel patterns are presented in Fig. 1. The mobility of the alpha-collagen bands appeared to be identical in samples obtained from normal control, pair-fed control, and lathyritic animals at all pH values observed. The alpha-collagen content of the samples obtained from lathyritic animals appeared to be increased in relation to control samples, and all types of alpha-chain appeared to be increased equally. There was no evidence for the presence of alpha-chains with significant alterations or of bands arising from the presence of chain fragments. The bands containing beta-collagen were decreased in size and staining intensity in the patterns of collagen obtained from lathyritic animals; however, there were no missing bands and the mobility did not appear to be altered.

A series of slowly migrating bands, labeled "X" in the figures, were observed in all patterns obtained below pH 5.0. The presence of these bands has been previously described in starch-gel patterns of normal collagen(15). Whether they contain dimers or larger units cannot be determined with certainty. Since 6 M urea was present, they are not likely to be non-specific aggregates. These bands and the beta-collagen bands are particularly well demonstrated in Fig. 2, a pattern obtained under conditions which permit high resolution of the more slowly migrating collagen species. The leading "X" band in the lathyritic collagen pattern is dramatically *increased* relative to control samples, suggesting that certain types of dimers or larger units are present in the denatured extracts of lathyritic tissues in *greater* than normal amounts. This observation has also been made on highly purified collagen samples extracted from the skins of lathyritic rats in dilute

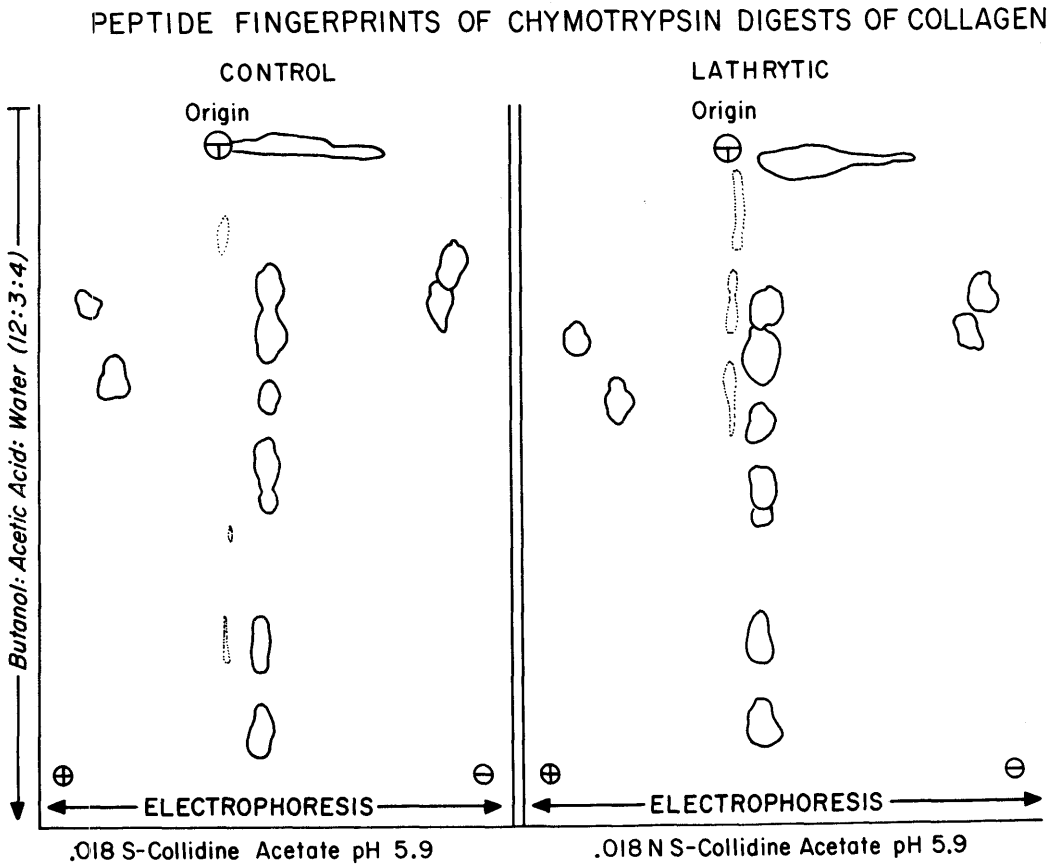

FIG. 3. Peptide fingerprints of chymotrypsin digests of collagen.

saline. The significance of the observation cannot presently be evaluated.

Starch-gel patterns were also obtained from collagen samples digested for various time periods with chymotrypsin. There appeared to be no difference in the susceptibility of normal and lathyritic collagen samples to enzyme digestion. Patterns obtained from material digested with chymotrypsin for peptide fingerprinting showed that under the conditions noted digestion was essentially complete and that discrete peptides were produced. The peptide fingerprints for control and lathyritic collagens are shown in Fig. 3. The enzyme and a small amount of undigested material remained at the origin. Two acidic, 2 basic, and 7 neutral peptides were observed. In addition, a series of very faintly ninhydrin-positive areas which did not migrate electrophoretically separated from the origin during the chromatographic phase. The positions of these areas, shown on the fingerprints by the dotted outlines, were variable from one run to another in both control and lathyritic collagen samples. The acidic and basic peptides were of special interest since they would be expected to contain the charged residues implicated in crosslinking. No significant differences were observed in the fingerprints of the lathyritic and control collagen digests.

The aldehyde content of the collagens obtained from the skins of normal control, pair-fed control, and lathyritic rats is shown in Table I. While the values obtained from normal collagen were about 0.7, those obtained using salt extracted lathyritic collagen were only about one-half this value. The yield of purified collagen obtained from the lathyritic skins in saline was elevated about 50% over controls and these extracts contained almost all of the excess soluble collagen. The alde-

TABLE I. Aldehyde Content of Collagen.*

Sample	μM of aldehyde per μM of collagen alpha-chain
.28M NaCl pair-fed	.66 ± .00
.28M NaCl control	.95 ± .09
.28M NaCl lathyritic	.30 ± .07
1.0 M NaCl pair-fed	.70 ± .01
1.0 M NaCl control	.73 ± .09
1.0 M NaCl lathyritic	.38 ± .14
1.0 M formic pair-fed	.69 ± .08
1.0 M formic lathyritic	.65 ± .05

* Method of Paz *et al*, reported values are the mean of 4 separate determinations except for acid collagens where 2 determinations were done, ±S.D.

hyde content of the acid extracted collagen, obtained from the lathyritic skins subsequent to salt extraction and containing no excess soluble collagen, was not different from that observed in comparable extracts of skins from normal animals.

Discussion. The collagen molecule is made up of 3 polypeptide chains, each of which appears to contain subunits(16,17). Recently reported evidence suggests that intramolecular and possibly intermolecular crosslinking occurs in N-terminal telopeptides located outside the helical portion of the molecule(7) and therefore easily susceptible to extrinsic influences. The type of structural alterations which might be induced by lathyrogens and result in an inability of the molecules to undergo crosslinking would be the absence of whole alpha-chains or subunits; deletion, addition, or substitution of critical amino acid sequences; or blocking of necessary functional groups. The techniques selected to demonstrate these possible intrinsic defects were starch-gel electrophoresis and peptide fingerprinting. Even though these techniques do not yield quantitative results they do appear to be the most sensitive procedures available for detection of minor structural alterations in polypeptide chains(18,19). However, these techniques did not demonstrate the presence of defects in lathyritic collagen molecules.

It is possible that a structural defect is present in the lathyritic alpha-chains but is obscured in the techniques used. This appears unlikely. The loss of altered chains or chain fragments was precluded by avoiding dialysis and purification of the extracts. The concentration of "affected" chains would be expected to be sufficiently high for demonstration since the soluble collagen yield from the lathyritic animals using the formic acid procedure was about 26% in excess of that obtained from control animals. Since the excess soluble collagen from lathyritic tissues gives rise almost exclusively to alpha-chains upon denaturation, it appears reasonable to expect that about half the alpha-collagen present in the denatured extracts is lathyritic.

In the electrophoretic technique used, the samples were run side by side so that small mobility differences could be observed. The alpha-chains were completely separated from the remainder of the sample and their mobility examined without the risk of alteration inherent in isolating pure alpha-collagen. Even though the starch-gel patterns would be expected to demonstrate only gross molecular alterations, the fingerprints permitted the examination of relatively small peptides in which minor charge alterations should be observable. Yet, no evidence for molecular alteration was seen.

Substances which are thought to be the covalent interchain crosslink of elastin and the intramolecular crosslink of collagen have recently been isolated and identified. These compounds derive from lysine and their formation appears to occur subsequently to aldehyde production by the oxidative deamination of lysyl side chains(7-9,20). Our data show that in salt extracted collagen from lathyritic skins, where the yield of soluble collagen is increased about 50% over controls, the aldehyde content is only about half that found in comparable control collagen. This suggests that the oxidative deamination of newly produced collagen chains is impaired in the lathyritic animals.

Taken together, these results imply that the lathyritic molecules are intrinsically normal and therefore capable of undergoing crosslinking, but they have not been oxidatively deaminated. The basic defect in lathyrism then seems to lie in the process which carries out oxidative deamination of collagen rather than in the alpha-chains *per se*.

Summary. Electrophoretic and peptide fingerprint studies carried out on formic acid

extracted collagen from the skins of control and lathyritic rats provided evidence that the excess soluble collagen molecules which accumulate in the tissues of lathyritic animals are intrinsically normal. But the lathyritic molecules are dramatically deficient in aldehyde content. Thus it appears that lathyrogens exert their effect on connective tissues by causing an inhibition of the oxidative deamination which appears to precede covalent crosslink formation in collagen and elastin.

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Amino Acid Composition of Three Immunological Types of Foot-and-Mouth Disease Virus. (31765)

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The amino acid compositions of only a few animal viruses have been determined, *e.g.*, polio, influenza, Shope papilloma and EMC viruses(1). The main reason for this is the difficulty in obtaining animal viruses in a state of purity in quantities adequate for meaningful analyses. We have described methods that yield essentially pure foot-and-mouth disease virus (FMDV) in milligram amounts from calf-kidney (CK) and baby-hamster kidney (BHK) cell cultures(2,3). This report contains the results of amino acid analysis of 3 immunological types of FMDV purified from tissue cultures.

Materials and methods. Virus. The immunological types of FMDV were type A, strain 119; type O, strain 9; and type C, strain 3. Type A_{119} virus was obtained from the Research Institute, Pirbright, England, as infectious bovine-tongue epithelial tissue. One portion was passed at Plum Island once in cattle, once in mice, and thereafter, approximately 150 times in primary CK cultures over a 12-year period. Large quantities of this virus were subsequently produced by single passages in uncloned BHK cell cultures in 2-liter roller bottles(3). Another portion of the bovine virus remained frozen for 12 years and was then passed 8 times in Povitsky-bottle cultures of CK cells. Hereafter, it is desig-