PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 174, 79–85 (1983)

Effect of 2-Aminoethyl-L-cysteine on Collagen Accumulation in Isolated Hepatic Granulomas[1] (41707)

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Abstract. Hydroxylysine residues are important structural components of collagen as sites of glycosylation and cross-linking. Because hydroxylysine is formed from lysine by post-translational enzymatic hydroxylation, we examined the effects of incorporation of a lysine analogue that could interfere with hydroxylation. For this purpose, we synthesized ^{35}S-labeled 2-aminoethyl cysteine. By addition of this compound to a smooth muscle cell culture, we obtained radiolabeled collagen which, upon acid hydrolysis, yielded the intact labeled analogue, thus demonstrating incorporation of the compound into collagen. Using a model hepatic collagen synthesizing system, freshly isolated granulomas derived from mouse livers after 8 weeks of infection with *Schistosoma mansoni,* incubation over a period of 7 days with [^{14}C]proline to measure collagen production, demonstrated a reduction in acid-precipitable [^{14}C]hydroxyproline per milligram DNA in granuloma cultures incubated with 2-aminoethyl cysteine compared to control cultures, and the effect was dose-related. That this decrease was selective for collagen accumulation over noncollagenous proteins was demonstrated by the finding of a significant reduction in the [^{14}C]hydroxyproline/[^{14}C] proline ratios compared to controls. A maximum incorporation of 10 residues of analogue/1000 residues was achieved. While the extent of hydroxylation of proline in collagen remained unchanged, hydroxylation of lysine decreased with increasing amounts of analogue incorporated. We conclude that in our system, 2-aminoethyl cysteine is incorporable into collagen. In a dose-related fashion, it selectively diminishes collagen accumulation compared to control.

Hepatic fibrosis is a process in which fibers are deposited in excess and interfere with liver function. These fibers are comprised mainly of the protein, collagen, and are produced, in general, as a response to liver injury. Because of its role in glycosylation (1) and cross-linking (2), hydroxylysine is an important structural component of collagen. It is formed by post-translational enzymatic hydroxylation of peptide-bound lysine residues in collagen and its formation is virtually unique to that protein. We were interested in determining whether or not collagen accumulation might be specifically decreased by the incorporation of a lysine analogue that might perform its functions like natural lysine with the exception that it would interfere with hydroxylation of the protein. Such an analogue might permit normal function of other proteins in which it has become incorporated but specifically alter the structure of collagen. A resultant decreased synthesis or increased susceptibility to degradation could result in a net decrease in collagen accumulation.

Materials and Methods. L-[^{35}S]Cysteine (specific activity, 1050 Ci/mmole) and [^{14}C]proline (specific activity, 285 mCi/mmole) were purchased from Amersham Company; ethyleneimine from K and K Chemical Company; and Dulbecco's Modified Eagle Medium (DMEM) and fetal calf serum (FCS) were products of GIBCO. Dowex resins were obtained from Biorad, carboxymethyl cellulose from Pharmacia. Highly purified bacterial Collagenase Form III was obtained from Advance Biofactures Corporation. All other reagents used were of the highest quality available.

Human uterine smooth muscle cells were maintained according to the method of Wu *et al.* (3).

Analysis of DNA was done according to the method of Kapascinski and Skoczylos (4). Hydroxyproline was quantitated by the method of Rojkind and Gonzalez (5).

CF1 female mice were infected with 50 cer-

[1] This work was supported in part by grants from the NIH AM-06573, Edna McConnell Clark Foundation 280-0059, and an American Liver Foundation Postdoctoral Fellowship Award. Present address: Department of Medicine, University of Connecticut School of Medicine, Farmington, Conn. 06032. We wish to thank Ms. Adele Giambrone for her technical assistance.

0037-9727/83 $1.50

cariae of a Puerto Rican strain of *Schistosoma mansoni* by the Department of Geographic Medicine, Case Western Reserve University, Cleveland, Ohio.

Amino acid analyses were performed on a Beckman 119 BL instrument employing a single-column system equipped with a split-stream attachment.

Preparation of the analogue. Chemical synthesis of 2-aminoethyl cysteine (AEC) was accomplished under conditions similar to that of Raftery and Cole (6) for aminoethylation of proteins. A fivefold molar excess of ethyleneimine was added to an aqueous solution of [^{35}S]cysteine and maintained for 2 hr at pH 9.0 by addition of 0.01 *N* HCl. The compound was purified by chromatography on a column (100 cm × 2.5 cm) of Dowex 50 ×8 maintained at 50°C and eluted with sodium acetate buffer 0.05 *M*, pH 6.5. Samples were collected and analyzed for radioactivity and ninhydrin reactivity. Tubes giving positive reactions were pooled and purity assessed by amino acid analysis.

Incorporability of analogue into collagen. A pure culture of smooth muscle cells which actively produce collagen was incubated at a density of 2×10^5 cells in DMEM, 10% FCS containing 0.1 m*M* 2-aminoethyl[^{35}S]cysteine, specific activity 356 Ci/mole, and 50 μg/ml ascorbic acid. After 5 days, the cells were washed and harvested. Collagen was isolated and purified (7) and examined by 7.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (8). The gel was sliced and radioactive bands determined by scintillation counting. Proof of analogue incorporation was achieved by amino acid analysis of an acid hydrolyzate of the purified collagen.

Liver granuloma preparation. The isolated granuloma culture of murine schistosomiasis was chosen as a model of fibrogenesis in liver fibrosis because of the demonstration that the vast majority of collagen production in this disease is associated with the granuloma (9). Isolated granulomas were prepared according to the method of Pellegrino (10) from livers of mice 7 to 8 weeks after infection. Following extensive washing, 0.5 ml settled volume of granulomas were incubated in 5 ml DMEM, 10% FCS containing ascorbic 50 μg/ml.

Effect of analogue on collagen accumulation by granuloma culture. Granulomas freshly isolated as described above were incubated in medium containing [^{14}C]proline and varying concentrations of AEC. At daily intervals, granulomas and medium were harvested, homogenized and precipitated with cold 10% trichloroacetic acid. A known amount of [^{3}H]proline-labeled collagen was added to calculate recovery. One portion of the homogenate was analyzed for DNA content to compensate for possible differences in the number of granulomas per analysis. The remainder was reconstituted to 70% ethanol to precipitate proteins. Pellets were recovered by spinning at 2000 rpm and extensively washed. Each pellet was hydrolyzed in 6 *N* HCl at 110°C under vacuum for 20 hr and analyzed for newly formed collagen present by quantitation of [^{14}C]hydroxyproline. In addition, to estimate general protein synthesis, the amount of peptide bound [^{14}C]proline was also determined.

AEC and hydroxylation of proline in collagen. Since the basis of our quantitation of collagen is the measurement of the amount of hydroxyproline produced, it was essential to determine whether or not the analogue affected extent of hydroxylation of proline in collagen.

To clarify this point, we made large-scale granuloma cultures incubated in varying concentrations of AEC for 7 days. Aliquots of granulomas, 5 mg/ml were homogenized in a Dounce homogenizer and digested with bacterial collagenase in the presence of 1 m*M* $CaCl_2$ at 37°C for 4 hr (11). Insoluble material was separated by centrifugation at 2000 rpm for 10 min. The supernatant was lyophilized, then hydrolyzed in acid and analyzed for hydroxyproline and proline as described above.

Hydroxylation of purified AEC-incorporated type I collagen. As a further confirmation, we isolated AEC-incorporated type I collagen from large scale cultures by pepsinization and carboxymethyl-cellulose chromatography according to the method of Chung *et al.* (7). The purified collagen was hydrolyzed and amounts of hydroxyproline, proline, lysine, hydroxylysine, and AEC were determined by amino acid analysis.

Results. *Preparation of aminoethyl cysteine (AEC).* Figure 1A shows an amino acid analysis of purified AEC. It demonstrates a single sharp peak appearing in the basic region elut-

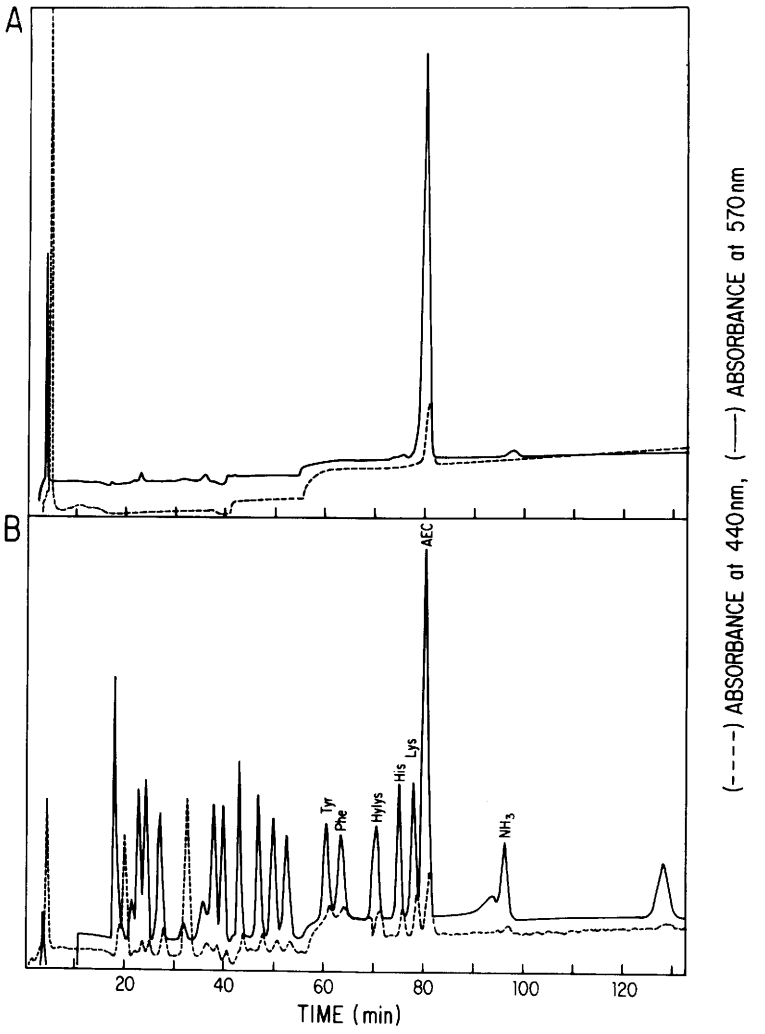

FIG. 1. An amino acid analysis of AEC following purification (A). Coinjection of AEC with a mixture of standard amino acids (B) illustrates the basic nature of this compound. In this analytic system, AEC is clearly distinguishable from lysine.

ing 2.5 min after lysine when coinjected with a mixture of standard amino acids, Fig. 1B. AEC is clearly distinguishable from lysine. Based on the amount of cysteine used for the reaction, a yield of 90% was obtained.

Incorporation of AEC into collagen. Figure 2 shows the radioactivity present in a SDS–polyacrylamide gel following electrophoresis of collagen derived from smooth muscle cells incubated in medium containing [^{35}S]AEC. An electrophoresis of mouse tail tendon type I collagen stained with Coomassie blue for comparison is shown in the upper portion of the figure. Radioactive bands correspond in position to α_1 and α_2 chains, the most rapidly migrating; β chains next, and γ chains barely entering the gel. The radioactivity corresponds exactly to the standard collagen chains and no other significant radioactivity is noted. While the presence of radioactivity in the protein bands indicates incorporation of radioactive material into protein, it does not prove incorporation of [^{35}S]AEC since the ^{35}S label could have been metabolized into another natural amino acid and incorporated to yield the radioactivity seen. Figure 3 shows an

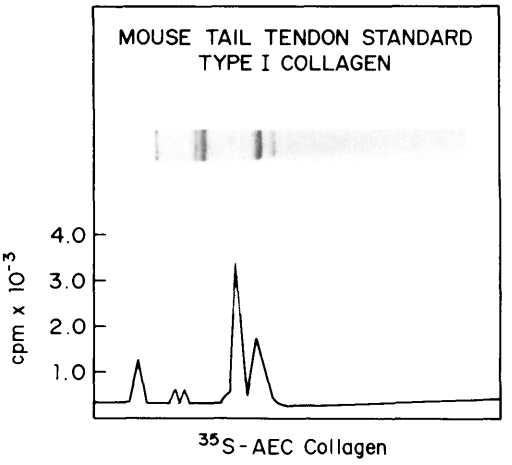

FIG. 2. A 7.5% SDS–polyacrylamide gel electrophoresis of purified [^{35}S]AEC-incorporated collagen derived from uterine smooth muscle cell cultures is shown in the lower portion of the figure. Radioactivity was determined by scintillation counting of 1.0-mm slices of the gel. A standard collagen sample stained with Coomassie blue is shown in the upper portion of the figure for comparison. The tops of the gels are aligned at the left.

amino acid analysis of an acid hydrolyzate of the purified collagen. The effluent stream from the instrument was split to permit collection of the eluate for scintillation counting. This demonstrated a single radioactive peak eluting 2.5 min after lysine in the position expected for AEC. Because no other radioactive amino acids were found, the results indicate that AEC was incorporated into collagen and it was solely responsible for the radioactivity seen in the gel. No conversion to other incorporable amino acids occurred under these conditions.

Effect of AEC on collagen accumulation by granuloma cultures. Figure 4 shows the effect of varying concentrations of AEC on collagen accumulation in isolated granuloma cultures as a function of time. Since hydroxyproline is virtually a specific marker for collagen, the conversion of [^{14}C]proline to acid precipitable [^{14}C]hydroxyproline was used to determine collagen production. To compensate for possible differences in the quantity of granulomas removed in each sample, DNA was measured and the hydroxyproline results expressed per

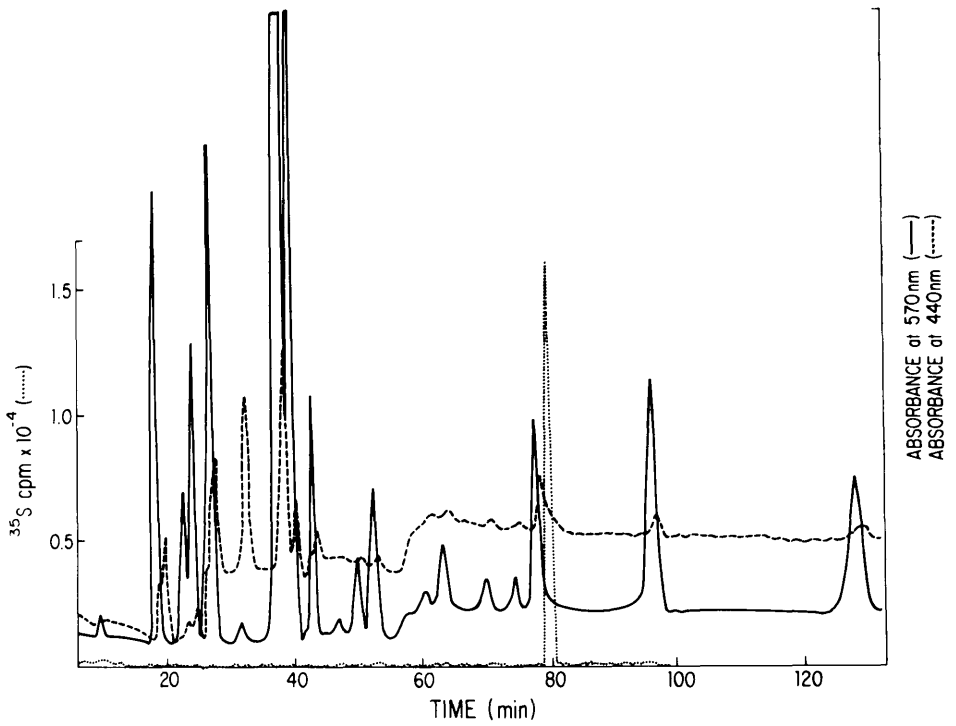

FIG. 3. An amino acid analysis of an acid hydrolyzate of purified [^{35}S]AEC-incorporated collagen. A fixed portion of the chromatographic stream was split to permit counting of radioactivity.

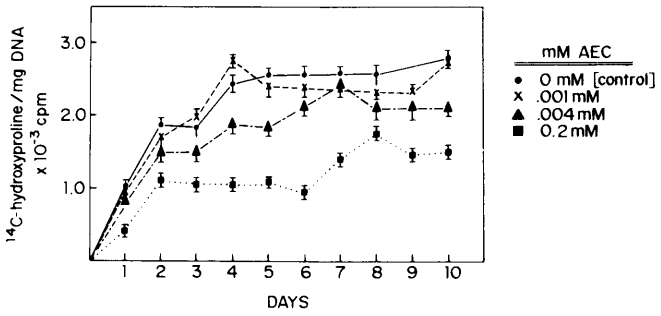

FIG. 4. Formation of [^{14}C]hydroxyproline by granuloma cultures as a function of time of incubation at various concentrations of AEC in the culture medium. Results are expressed as a ratio of [^{14}C]hydroxyproline per milligram of DNA in each sample to compensate for possible differences in granuloma numbers in each analysis.

milligram DNA. Hydroxyproline values of the control cultures increased steadily reaching a plateau by Day 5. At 0.001 m*M* AEC, there was no significant difference compared to control. However, at 0.004 m*M* and above, there was a significant decrease in both the rate and maximum collagen accumulation. The results indicate a dose related response in the appearance of [^{14}C] hydroxyproline/ milligram DNA. However, this could simply be due to a decrease in general protein production and not be specific for collagen. To investigate this possibility, the amount of acid-precipitable [^{14}C]proline was determined as a measure of general protein formation. In this way, the relation of collagen production to total protein production could be expressed as a ratio of [^{14}C]hydroxyproline/[^{14}C]proline shown in Fig. 5. The control cultures had no significant difference in the ratio until the fifth day when there was a small decrease in the proportion of [^{14}C]hydroxyproline to [^{14}C]proline. However, the ratio was significantly lower in all cultures containing AEC. In addition, cultures with AEC present had a steady decrease in the ratios indicating a progressive preferential decrease in the contribution of collagen compared to general proteins in cells grown in the presence of AEC.

AEC and hydroxylation of proline in collagen. Table I shows the imino acid composition of hydrolyzed samples from collagenase

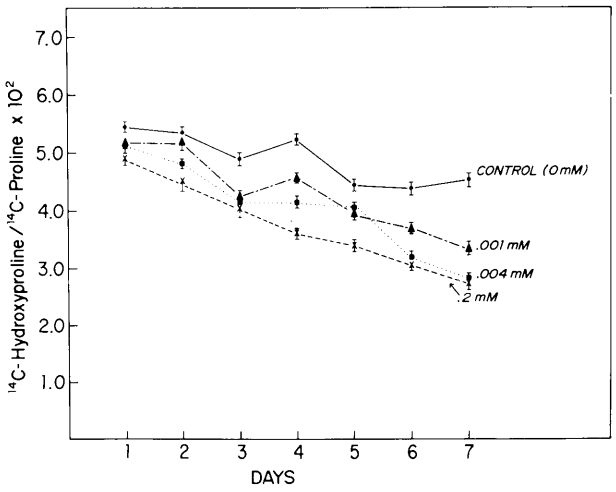

FIG. 5. A plot of the ratio of [^{14}C]hydroxyproline to [^{14}C]proline in newly synthesized acid-precipitable protein as a function of time of incubation in various concentrations of AEC in the granuloma culture medium.

TABLE I. IMINO ACID CONTENT[a,b] OF COLLAGENASE DIGESTS OF NORMAL AND AEC-TREATED GRANULOMA CULTURES

	Concentration of AEC in medium (m*M*)			
	0	0.001	0.01	0.1
Hydroxyproline	67 ± 3	64 ± 4	65 ± 3	61 ± 6
Proline	142 ± 7	137 ± 8	145 ± 7	136 ± 10
Hydroxyproline/hydroxyproline + proline	0.47 ± 0.03	0.46 ± 0.03	0.46 ± 0.03	0.49 ± 0.04

[a] Mean ± 1 SD of triplicate experiments.
[b] Residues per 1000 amino acid residues.

digests of AEC-incorporated collagens. There was no significant difference in the extent of hydroxylation of proline residues in control collagen compared to AEC-incorporated collagen.

Because collagenase digestion assays total collagenous protein and does not involve separation of proteins by precipitation, the data indicate that AEC-incorporated collagens do have normally hydroxylated proline and the TCA precipitation used in previous assays for peptide-bound hydroxyproline did not reflect artifactually high hydroxyproline/proline ratios due to loss of underhydroxylated collagens as TCA soluble peptides.

Hydroxylation of purified AEC-incorporated type I collagen. Table II shows the results of amino acid analyses of acid hydrolyzates of purified type I collagen from granuloma cultures formed in the presence of varying concentrations of AEC. The amount of AEC incorporated increased with increasing AEC concentration in the medium and reached a maximum of 10 residues/1000 residues. In the granuloma cultures treated with 0.01 and 0.1 m*M* AEC, the total lysyl residues (lysine plus hydroxylysine) decreased significantly. In each instance, the decrease was accounted for, within the limits of error, by the number of AEC residues incorporated suggesting substitution of AEC for lysine residues in the collagen molecule. The results, as in the collagenase digests, indicate that the extent of hydroxylation of proline remained constant at 47% in spite of increasing amounts of AEC incorporated. Our calculations based on the assumption of a constant amount of hydroxyproline present per molecule of collagen were, therefore, valid. In contrast, the extent of hydroxylation of the natural lysine residues in AEC-incorporated collagen decreased significantly from 38 to 17% with increasing amounts of AEC incorporated. No unusual peaks appeared in the basic amino acid regions of these analyses indicating that no detectable hydroxylation of AEC had occurred.

Discussion. Our results demonstrate the ability of the lysine analogue, 2-aminoethyl cysteine (AEC), to become incorporated into proteins resulting in a selective decrease in

TABLE II. SELECTED AMINO ACID CONTENT[a,b] OF AEC-INCORPORATED TYPE I COLLAGENS

	Concentration of AEC in medium (m*M*)			
	0	0.001	0.01	0.1
AEC incorporated	0	0.6 ± 0.1	3.5 ± 0.3	10 ± 0.5
Hydroxyproline	94 ± 6	90 ± 5	84 ± 6	88 ± 6
Proline	106 ± 7	110 ± 6	116 ± 6	112 ± 5
Hydroxyproline/hydroxyproline + proline	0.47 ± 0.03	0.45 ± 0.03	0.42 ± 0.03	0.44 ± 0.03
Lysine	25 ± 1	25 ± 1	27 ± 2	25 ± 1
Hydroxylysine	15 ± 1	14 ± 1	9 ± 1	5 ± 1
Hydroxylysine/lysine + hydroxylysine	0.38 ± 0.05	0.35 ± 0.03	0.26 ± 0.03	0.17 ± 0.02

[a] Mean ± 1 SD of triplicate experiments.
[b] Residues per 1000 amino acid residues.

accumulation of intact collagen versus general proteins in our model of hepatic fibrosis, the murine schistosomal granuloma system. Our results confirm previous data on procollagen synthesis in chick tendon fibroblasts (12). In those experiments, however, actual incorporation of the analogue was inferred but not proven.

On the basis of the demonstration that granulomas are responsible for 90% of the total collagen production in the schistosome-infected mouse liver (9), our finding of inhibition of collagen accumulation of isolated granulomas is likely to represent an inhibition of the majority of the collagen synthesizing capability of these granulomatous livers.

The mechanism of the effect is presently under investigation. One possibility that particularly interests us is that of increased susceptibility of the collagen to proteolytic digestion. This may be due to changes in the helical structure induced by the incorporation of the analogue. Several proline analogues have been shown to produce abnormalities in collagen helical structure by causing decreased hydroxylation of proline (13,14). These collagens were found to be more susceptible to proteolysis.

Although there is no evidence that indicates that either lysine or hydroxylysine residues are involved in maintaining the collagen helix, it is possible that, because of the abnormal side chain, the presence of the analogue itself, may induce local aberrations in the helical regions adjacent to site of incorporation enhancing susceptibility to proteolysis. In this regard, X-ray crystallographic data could be helpful in assessing the structural effects of AEC incorporation.

Another lysine analogue, 4,5-dehydrolysine, has been studied with respect to effects on collagen structure. As in the experiments on proline analogues, 4,5-dehydrolysine-incorporated collagens were found to be more susceptible to pepsin (13). However, here too, hydroxylation of proline was diminished in these molecules so that the effect of the analogue itself on the collagen helix could not be studied independent of the effect of underhydroxylation.

If further experimentation demonstrates selectivity for inhibition of collagen accumulation during fibrogenesis *in vivo,* AEC could prove to be of significant value therapeutically in preventing the presently inexorable progression of this pathological process in man.

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Received February 7, 1983. P.S.E.B.M. 1983, Vol. 174.