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THE INCORPORATION OF [^{14}C]GLYCINE INTO THE SUBUNITS OF COLLAGENS FROM NORMAL AND LATHYRITIC ANIMALS

G. R. MARTIN, KARL A. PIEZ AND MARC S. LEWIS

National Institute of Dental Research, National Institutes of Health, Bethesda, Md. (U.S.A.)

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SUMMARY

The incorporation of [^{14}C]glycine into the subunits of skin collagen was followed in normal and lathyritic rats. Label appears first and at the same rate in the primary subunits, $\alpha 1$ and $\alpha 2$, and later in $\beta 1$ and $\beta 2$. This is explained by the fact that $\beta 1$ and $\beta 2$ are derived from $\alpha 1$ and $\alpha 2$ by intramolecular crosslinking. In lathyritic animals the formation of $\beta 1$ and $\beta 2$ is markedly reduced though the synthesis of collagen proceeds. This defect is evident long before the appearance of the gross symptoms of lathyrism. The results are consistent with the suggestion that the lack of intramolecular crosslinks affects the integrity of connective tissue through a loss of fibril stability resulting in the gross symptoms characteristic of lathyrism.

INTRODUCTION

The administration of a number of simple compounds such as β-aminopropionitrile to growing animals induces the condition known as lathyrism which is characterized by a variety of connective tissue malformations such as exostoses, hernias and aneurysms. Accompanying these symptoms is a general weakening of tendons and ligaments and an increased tissue fragility (see ref. 1 for recent bibliography). A greatly increased extractability of collagen in cold neutral salt solution has been found by LEVENE AND GROSS[1] to parallel this condition in chick embryos. They concluded that lathyrogenic agents affect in some undefined manner the intermolecular aggregation within the collagen fibril.

Recent chromatographic investigations[2,3] of the intramolecular structure of normal collagen have demonstrated that the newly formed collagen molecule (neutral salt-extracted) is composed of three subunits of similar size representing at least two types (designated $\alpha 1$ and $\alpha 2$) which differ in amino acid composition. Cold 0.5 M acetic acid extracts an older collagen which contains in addition to $\alpha 1$ and $\alpha 2$, two larger components (designated $\beta 1$ and $\beta 2$) apparently formed by a maturation process involving the formation of intramolecular covalent crosslinks. $\beta 1$ is composed of an $\alpha 1$ and an $\alpha 2$ subunit and $\beta 2$ is composed of two $\alpha 1$ subunits. In contrast to normal collagen, acid-extracted collagen from lathyritic animals has been found to contain a very small proportion of $\beta 1$ and $\beta 2$ (see ref. 4). It was suggested that this represents a

Abbreviation: BAPN, β-aminopropionitrile.

molecular defect in the maturation process and is related to the gross symptoms of lathyrism.

The present report is concerned with a study of the formation of β_1 and β_2 from α_1 and α_2 by following the incorporation of [^{14}C]glycine into the subunits of normal and lathyritic collagen. The amino acid composition of lathyritic collagen and its subunits was also determined. A portion of these results have appeared in a preliminary report[1].

EXPERIMENTAL

Isotope incorporation experiments

In Expt. 1 (Table I), 40 rats (100 g male Sprague–Dawley) were maintained on a low protein diet[5] with and without the intraperitoneal injection of 100 mg of BAPN per day. On the third day, 10 control and 10 BAPN-treated animals received 25 μC [2-^{14}C]glycine. These animals (acute toxicity group) were sacrificed 24 h later. The other animals were continued on the diet with and without BAPN for 34 days. On the 33rd day they received 25 μC of [2-^{14}C]glycine. These animals (chronic toxicity group) were sacrificed 24 h later.

In Expt. 2, 18 (100 g male Sprague–Dawley) rats were maintained on an adequate diet for thirty days with and without the intraperitoneal injection of 100 mg BAPN per day. On the 30th day all animals received 50 μC/100 g of [1-^{14}C]glycine. The control animals weighed an average of 175 g and the lathyritic animals averaged 155 g at this time. Three normal and 3 BAPN-injected animals were sacrificed on days 1, 3 and 7 following the [1-^{14}C] glycine administration.

In Expt. 1, collagen was extracted and purified as described[4]. In Expt. 2, collagen was obtained from the coarsely ground skins by a single extraction with at least a tenfold excess (v/w) of 0.5 M acetic acid at 5° for 48 h. The collagen solubilized by this procedure was purified by alternate salt precipitation at low pH and low ionic strength precipitation at neutral pH (see ref. 3) and lyophilized from acetic acid solution to give a dry material. Weighed amounts of these samples were then taken for examination in the ultracentrifuge, chromatography, and amino acid analysis.

Chromatography and ^{14}C assay

Collagen samples weighing about 50 mg were denatured at 40° and chromatographed on CM-cellulose as described[3,6]. The radioactivity in each 10-ml fraction from the column was assayed for ^{14}C activity by plating and drying 1-ml portions on 1.25-in planchets containing a circle of lens paper and counting in a low background (1.6 counts/min) gas flow counter. Each sample was counted for 1000 counts or 50 min whichever came first. In some experiments, fractions were taken from the α_1, α_2 and β_1 chromatographic peaks and protein was isolated by gel-filtration[3]. The [^{14}C]glycine activity in hydrolysates of the isolated subunits was measured in the effluent from the automatic amino acid analyzer with a scintillation flow counter[7].

Amino acid analyses

Samples of purified collagen or fractions isolated from the chromatographic effluents were hydrolyzed with 6 N HCl and analyzed as previously described[6] employing an automatic amino acid analyzer[8].

Sedimentation velocity

Denaturated collagen was examined in a Spinco Model E ultracentrifuge employing Schlieren optics. Native collagens were dissolved by stirring overnight in sodium formate buffer (pH 3.75, $I = 0.15$) at 5°. They were denatured by warming to 4° just before being placed in the cells.

RESULTS

As previously reported[4], samples of denatured skin collagen from chronically lathyritic rats (34 days BAPN) when sedimented in the ultracentrifuge show a large peak corresponding to $\alpha 1 + \alpha 2$ and only a small peak representing the heavier components $\beta 1 - \beta 2$, while normal acid-extracted collagen contains about equal amounts of the two weight classes. Collagen from acutely toxic animals (4 days BAPN) has essentially a normal pattern, although some increase in α_1 and α_2 may be indicated. Fig. 1 shows ultracentrifuge patterns of typical samples which illustrate these results. The interpretation of the sedimentation patterns is complicated by the fact that the JOHNSTON–OGSTON effect[9] is pronounced under these conditions. In normal, acid-extracted collagen, each peak actually represents about half the weight of the sample as determined from chromatographic studies (see below). The effect increases with increasing protein concentration. Therefore, quantitative results are more easily obtained from the chromatograms.

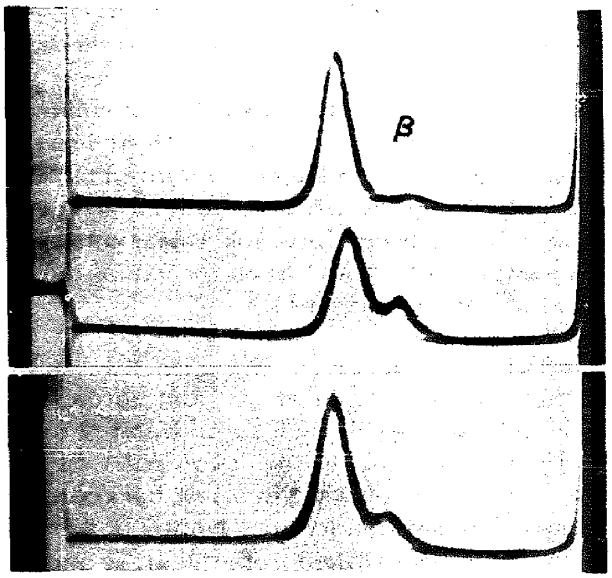

Fig. 1. Sedimentation patterns of denatured, acid-extracted rat-skin collagen. Upper pattern (wedge-window cell), 0.7% collagen from chronically lathyritic rats; middle pattern (standard cell), 0.6% collagen from normal rats; lower pattern (separate experiment), 0.7% collagen from acutely lathyritic rats. $\alpha = \alpha 1 + \alpha 2$, $\beta = \beta 1 + \beta 2$. Sodium formate buffer (pH 3.75, I = 0.15), 128 min at 59780 rev./min, at 40°, 12 mm Kel-F cells, 65° bar angle, sedimentation from left to right.

Typical chromatograms obtained in Expt. 2 showing both protein concentration and ^{14}C activity are reproduced in Fig. 2*. In addition to the lower proportion of $\beta 1$ and $\beta 2$ in collagen from lathyritic animals also seen in the ultracentrifuge, it is readily apparent that 7 days after the administration of [^{14}C]glycine there is considerably less incorporation of ^{14}C into $\beta 1$ and $\beta 2$ than $\alpha 1$ and $\alpha 2$ in the case of the lathyritic animals.

* The resolution in these chromatograms is not as good as obtained in other experiments[2,4] though it is sufficient for the purposes of the present studies. This is typical of the variability observed.

Specific activities were calculated by estimating the shape of the concentration and radioactivity curves and calculating total counts and total weight of protein (using an extinction coefficient of 20 at 230 mμ (see ref. 3)) for each subunit. The results appear in Tables I (Expt. 1) and II (Expt. 2). The specific activities of β2 are subject to a large error owing to the small amount present, its low specific activity, and the tailing of α1 into β2. For this reason the β2 data were given less consideration.

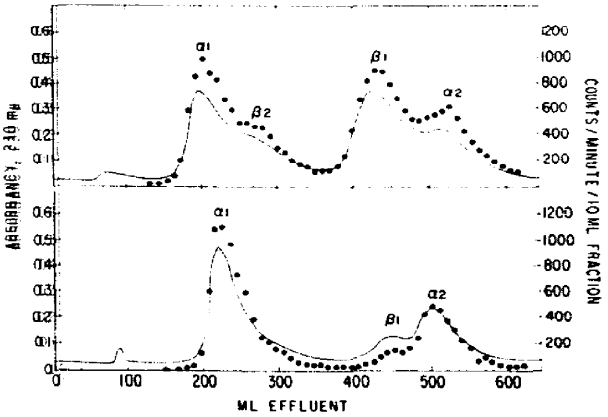

Fig. 2. Elution patterns of denatured rat-skin collagen chromatographed on CM-cellulose at 40°. Upper chromatogram, collagen from normal animals; lower chromatogram, collagen from chronically lathyritic animals (Expt. 2) 7 days after administration of [^{14}C]glycine. Solid line, absorbancy at 230 mμ; ●, counts/min in each 10-ml fraction.

Although the data show considerable variability, it is likely that α1 and α2 have the same specific activity. This can be determined with more confidence from the more precise data which were obtained for the 1-day samples in Expt. 2 by direct analysis of the glycine (see footnote of Table II). Although the values obtained for α1 and α2 from normal collagen are significantly different in a statistical sense ($p < 0.05$), the difference is probably not real since the α2 sample may have contained some β1 which overlaps it (see Fig. 2) and has a much lower specific activity. The specific activities of α1 and α2 from lathyritic collagen do not differ. Since β1 and β2 are derived from α1 and α2, β1 and β2 must also have the same specific activity if it is assumed that they are formed at the same rate. Since higher values were found for β2, it is probable that the degree of tailing of α1 into β2 was underestimated.

TABLE I

^{14}C ACTIVITY IN THE SUBUNITS OF SKIN COLLAGEN FROM NORMAL AND LATHYRITIC RATS ONE DAY AFTER ADMINISTRATION OF [^{14}C]GLYCINE

	Maximum (4 days BAPN)							
	Counts/min/mg protein				*Percent total counts*			
	α1	α2	β1	β2*	α1	α2	β1	β2*
Normal	902	790	236	(198)	52	26	17	(5)
Lathyritic	256	196	44	(27)	68	26	4	(2)
	Chronicity (34 days BAPN)							
Normal	365	345	74	(115)	55	26	12	(7)
Lathyritic	244	204	22	(49)	68	28	2	(2)

* These values are approximations owing to the small number of counts represented and contamination of β2 with α1.

TABLE II

^{14}C ACTIVITY IN THE SUBUNITS OF SKIN COLLAGEN FROM NORMAL AND CHRONICALLY LATHYRITIC RATS AT SEVERAL TIMES AFTER ADMINISTRATION OF [^{14}C]GLYCINE

Days after [^{14}C]glycine	Counts/min/mg protein			
	$\alpha 1$	$\alpha 2$	$\beta 1$	$\beta 2$*
Normal				
1**	205	210	86	(123)
3	193	215	135	(170)
7	147	170	129	(151)
Lathyritic				
1**	160	180	45	—
3	143	143	68	—
7	138	128	85	—

* These values are approximations owing to the small number of counts represented and contamination of $\beta 2$ with $\alpha 1$. Values for $\beta 2$ in lathyritic collagen were not estimated, but represented less than 5% of the weight and 2% of the counts.

** The [^{14}C]glycine activity was determined directly in acid hydrolysates of the subunits from the 1-day group. The following values were obtained (disintegrations/min/0.2 μmole glycine $\pm$ standard error). Normal: $\alpha 1$, 209 $\pm$ 4; $\alpha 2$, 191 $\pm$ 5; $\beta 1$, 85 $\pm$ 3. Lathyritic: $\alpha 1$, 164 $\pm$ 4; $\alpha 2$, 172 $\pm$ 5; $\beta 1$, 46 $\pm$ 3.

In Expt. 1 (Table I) the specific activity of $\beta 1$ was about one quarter that of $\alpha 1$ and $\alpha 2$ in the normal animals while in the lathyritic animals the ratio was less than one tenth. This is particularly evident by comparison of the proportions of total counts appearing in $\beta 1 + \beta 2$. In normal animals $\beta 1 + \beta 2$ represented about 20% of total counts incorporated but only about 5% in lathyritic animals. This was true whether the animals were acutely toxic, showing few of the gross symptoms of lathyrism and an essentially normal distribution of subunits, or chronically toxic.

Data from Expt. 2 (Table II and Fig. 3) show the conversion of $\alpha 1$ and $\alpha 2$ to $\beta 1$ and $\beta 2$ as a function of time. In the normal animal, the ratio of specific activities $(\alpha 1 + \alpha 2)/(\beta 1 + \beta 2)$ was about 2 at 1 day, less than 2 at 3 days, and nearly 1 at 7 days.

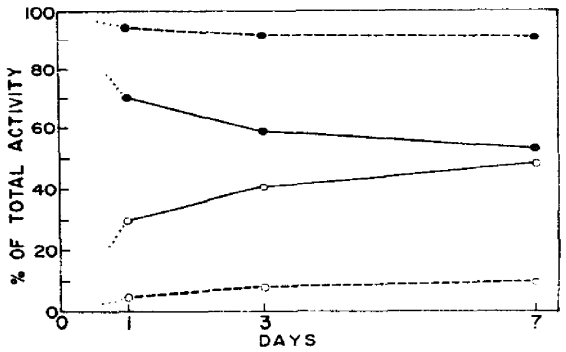

Fig. 3. The incorporation of ^{14}C into the subunits of rat-skin collagen at various times after the administration of [^{14}C]glycine. ———, normal animals; – – – –, lathyritic rats; ●—●, $\alpha 1 + \alpha 2$; ○—○, $\beta 1 + \beta 2$.

These ratios were larger in lathyritic animals, indicative of a markedly decreased formation of $\beta 1$ and $\beta 2$. Since the specific activity data do not reflect the smaller proportions of $\beta 1$ and $\beta 2$ in collagen from lathyritic animals compared to normal animals and since the specific activities may be affected by other variables which are uncontrolled (such as the proportion of total collagen in the tissue represented by the

sample and growth rate of the animal), the results can be more easily interpreted when expressed as percent of total counts in the sample. When this is done (Fig. 3), the expected precursor–product relationship ($\alpha \rightarrow \beta$) is evident. However, the rate of transformation differs greatly in the two groups. In the normal animal, nearly 50 % of the counts are in $\beta 1$ and $\beta 2$ after 7 days, while in the lathyritic animal, nearly all the activity remains in $\alpha 1$ and $\alpha 2$, less than 10 % appearing in $\beta 1$ and $\beta 2$ after 7 days.

The weight ratios $\alpha 1/\alpha 2$ calculated from the chromatograms were always close to 2.0 for collagens from both normal and lathyritic rats. The ratio of $(\alpha 1 + \alpha 2)/(\beta 1 + \beta 2)$ in skin collagen from acutely toxic animals was very close to normal values, changing from about 0.9 to 1.1. In collagen from chronically toxic animals this ratio increased to about 3 (see Table I of ref. 4).

Fractions were taken from the peak regions of $\alpha 1$, $\beta 1$ and $\alpha 2$ on chromatograms of collagens from the 1-day groups of normal and lathyritic rats in Expt. 2. The protein was isolated and analyzed for amino acid content (Table III) and specific activity of the glycine (see footnote of Table II). Some of the minor differences which appear in this set of values are within the expected experimental error for single analyses[8]. It is concluded that the subunits from normal and lathyritic collagen do not differ significantly in amino acid content. In both instances $\beta 1$ had a composition equivalent to an equal mixture of $\alpha 1$ and $\alpha 2$. $\beta 2$ was not present in sufficient quantity in lathyritic collagen to be isolated but presumably had the same composition as $\alpha 1$ as in the case

TABLE III

AMINO ACID COMPOSITION* OF THE SUBUNITS OF SKIN COLLAGEN FROM NORMAL AND LATHYRITIC RATS

	Residues/1000 total residues					
	Normal			*Lathyritic*		
	$\alpha 1$	$\alpha 2$	$\beta 1$	$\alpha 1$	$\alpha 2$	$\beta 1$
3-Hydroxyproline	1.2	0	0.8	0.9	0	0.7
4-Hydroxyproline	92	86	87	90	78	84
Aspartic acid	46	43	45	46	44	45
Threonine	20	18	20	20	20	19
Serine	38	41	42	41	43	41
Glutamic acid	73	70	71	74	70	70
Proline	130	116	123	129	115	123
Glycine	335	336	330	334	333	332
Alanine	110	102	108	112	103	107
Valine	21	33	28	20	34	28
Methionine	7.8	5.6	6.7	8.7	6.1	7.2
Isoleucine	6.5	16	12	6.3	17	12
Leucine	18	32	26	18	33	26
Tyrosine	1.9	2.6	2.0	2.4	2.7	2.3
Phenylalanine	13	11	11	12	11	11
Hydroxylysine	4.2	7.4	6.0	4.1	7.8	6.2
Lysine	30	22	26	31	23	26
Histidine	2.0	7.7	5.0	2.1	8.9	5.3
Arginine	50	51	52	50	52	52
Amide N**	(42)	(52)	(43)	(37)	(43)	(46)

* Single analyses of protein obtained from the peak regions of the chromatogram reproduced in Fig. 2 are presented.

** Some of the amide N values appear to be somewhat high, resulting perhaps from contamination. Values near 40 are usually obtained[3].

of normal collagen[2,3]. The unfractionated collagens had compositions equivalent to two $\alpha 1$ plus one $\alpha 2$. These data are consistent with the structural relationships outlined above. The presence of a new amino acid, 3-hydroxyproline, is discussed elsewhere[3].

DISCUSSION

That α subunits are labeled at a faster rate than β was first demonstrated by OREKOVITCH *et al.*[10,11] working with ammonium sulfate fractions of denatured collagen. It is now possible to explain this rather puzzling result in a conclusive manner on the basis of the demonstration[2,3,12] that $\beta 1$ and $\beta 2$ arise from the primary subunits $\alpha 1$ and $\alpha 2$ after their assembly into a native molecule and during or after their incorporation into fibrils. That is, a sample of collagen contains, in general, more than one species of molecule. These include recently synthesized molecules which are not internally crosslinked and at least two kinds of molecules which are internally crosslinked*. Therefore when a pulse of [^{14}C]glycine is introduced, after a short time (less than 1 day in the case of rat skin) most of the label will be in $\alpha 1$ and $\alpha 2$. At longer time periods $\beta 1$ and $\beta 2$ become more heavily labeled and eventually, after a sufficiently long time, $\beta 1$ and $\beta 2$ would presumably have even higher specific activities than $\alpha 1$ and $\alpha 2$. In lathyritic rats this course of events is drastically slowed. This does no rule out the possibility that $\beta 1$ and $\beta 2$ formed prior to the administration of BAPN are to some extent reconverted to $\alpha 1$ and $\alpha 2$. Whether or not there is any direct effect of the toxic agent on the rate of collagen synthesis cannot be determined from the present studies, but at least some new collagen is made even in chronically toxic animals. This new collagen is normal with respect to its amino acid composition.

Of some importance is the demonstration that a decreased rate of formation of $\beta 1$ and $\beta 2$ in lathyritic animals is evident from the labelling studies almost immediately after the start of BAPN administration at a time when gross symptoms of lathyrism are not evident. This indicates that the action of the lathyrogenic agent is immediate and suggests that the disruption of intramolecular crosslinks or interference with their formation may be the primary effect of the lathyrogenic agent. Whatever the mechanism of the effect, it is reasonable to speculate that the intramolecular crosslinking process is related to perfection of fibril structure through the formation of intermolecular crosslinks of a covalent character. It is also possible that the intramolecular crosslinking is necessary to stabilize the molecular structure. In the absence of crosslinking, the collagen might be more susceptible to denaturing conditions and enzymic attack. In either case it is likely that collagen fibrils would not attain their optimal tensile strength which would interfere with the function of the tissue and result in the gross symptoms characteristic of lathyrism.

ACKNOWLEDGEMENT

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* In addition to molecules containing $\beta 1$ or $\beta 2$, a small proportion is present in which all three α subunits are joined. This is referred to as the γ-component (see refs. 3 and 12).

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