The Effect of Semicarbazide on the Nature and Stability of Collagen Fibrils

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Semicarbazide affected both the final width and stability of fibrils reconstituted from solutions of acid-soluble collagen. Fibril width was increased after semicarbazide treatment at pH 2.6 and 4.3, whereas after treatment at pH 8.9 it decreased. Fibril stability was decreased after semicarbazide treatment at all values of pH and temperature, indicating the inhibition of intermolecular cross-linking. A direct binding of semicarbazide to the $\alpha\beta$ -unsaturated aldehyde groups in the intramolecular cross-link was demonstrated.

The reconstitution of native-type fibrils from solutions of soluble collagen can be effected *in vitro* by a change of temperature (Gross, 1956) or pH (Wood & Keech, 1960). The time-course of fibril formation, which may be followed turbidimetrically, consists of a lag phase during which no fibrils are observed followed by a sigmoid growth phase. The two phases result from the aggregation of different collagen fractions, nucleus-forming and growth collagen (Wood, 1962). This heterogeneity with respect to fibril formation is related to subunit composition, nucleus-forming collagen exhibiting a higher β/α subunit ratio than growth collagen. The final extinction value is determined by the nucleation process, and it can be shown that there is a correlation between this value and both the extent of precipitation and the structure of the fibrils formed (Wood & Keech, 1960).

Electrostatic forces and hydrogen bonds are important for the specific alignment and partial stabilization of the individual molecules within the native-type fibril (Gross & Kirk, 1958; Bensusan, 1960; Wood & Keech, 1960; Neumark & Marót, 1966). However, the main stabilizing forces are hydrophobic, stabilization arising from a gain in entropy due to the expulsion of organized water molecules that surround the collagen molecules within the fibril (Cassel, 1966; Fessler, 1966; Cassel & Christensen, 1967; Grant & Alburn, 1968). Further stabilization occurs by the formation of covalent intermolecular cross-links, since the fraction of reconstituted fibrils that will dissolve on cooling (Fessler, 1960*a,b*; Wood, 1962) decreases with increased incubation of fibrils at 37°C (Gross, 1958*a,b*).

Lathyrism, which can be experimentally induced

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in animals by the administration of certain nitriles, ureides, hydrazides and hydrazines, results in a collagen deficient in intramolecular cross-links (Martin, Piez & Lewis, 1963). Moreover, solutions of lathyrin collagen do not form stable fibrils at 37°C (Gross, 1963*a,b*), indicating a lack of intermolecular cross-linking. Attempts have been made to simulate the action of lathyrogens *in vivo* by studying their effect *in vitro* (Fessler & Bailey, 1966; Wood, 1963), but the results have varied considerably. The action of semicarbazide on the formation *in vitro* of fibrils from acid-soluble collagen solutions was shown to be pH- and temperature-dependent (Convy & Wynn, 1966*a*, 1967). The present paper describes the effect of semicarbazide on the nature and stability of the reconstituted fibrils and compares the results with those of other studies *in vitro*.

MATERIALS AND METHODS

Preparation and treatment of acid-soluble collagen solutions. Acid-soluble collagen solutions (concentration 0.16–0.24%), pH 4.2 (prepared from calf dermis), were treated with an equal volume of solutions containing excess of semicarbazide hydrochloride or NaCl at various temperatures and pH values, and exhaustively dialysed as previously described (Convy & Wynn, 1967).

Turbidimetric study of fibril formation and fibril stability. Fibril formation from duplicate samples of the non-diffusible material of each semicarbazide- or NaCl-treated solution (test and control respectively) was followed turbidimetrically at pH 7 at 25°C by monitoring the increase in E_{400} (Convy & Wynn, 1967), and the precipitated fibrils were incubated at 25°C for 22 h. Duplicate samples of untreated collagen solutions were similarly precipitated and incubated at 25°C. After incubation, one of each duplicate test and control precipitates was immediately centrifuged at 4000g for 30 min and the supernatant fraction was analysed for hydroxyproline by the method of Woessner (1961). The duplicate test and

control precipitates were cooled to 4°C and the decrease in E_{400} was recorded with time until constant. The cooled precipitates were then centrifuged at 4000g for 30 min and the supernatants were analysed for hydroxyproline.

Quantitation of the extent of fibril formation and fibril stability. The fractions of total collagen originally present in solution that (a) were precipitated at 25°C, (b) resisted dissolution and (c) were solubilized at 4°C were calculated from the hydroxyproline analyses of the original solution at zero time of precipitation and the supernatant fractions before and after cooling. The fraction solubilized at 4°C was also calculated from the expression:

$$(E_{\infty(25)} - E_{\infty(4)})/E_{\infty(25)}$$

where $E_{\infty(25)}$ is the final extinction before cooling and $E_{\infty(4)}$ is the extinction after cooling at 4°C.

U.v. difference spectra. Difference spectra between the non-diffusible material of the test and control solutions of identical concentration were recorded at room temperature before fibril formation (Convy & Wynn, 1967). The extinction of each solution in the 220–225 nm region varied between 1.8 and 3.0, the absolute value depending on the protein concentration (0.08–0.12%) and mode of

treatment. The stray-light level was found to be less than 0.1% at wavelengths above 210 nm.

RESULTS

Turbidimetric observations. Fig. 1 illustrates the time-course of fibril formation and dissolution for untreated collagen, and for collagen solutions treated with semicarbazide (tests) and sodium chloride (controls) at a given temperature at pH 2.6, 4.3 and 8.9. Similar patterns were observed after treatment at other temperatures at each pH value. However, after treatment above 32°C at pH 2.6, fibril formation was negligible and could not be studied. For all pH values the fibrils formed from the test solutions dissolved more readily on cooling than those formed from the controls, as shown by the greater decrease in extinction. This instability was independent of the magnitude of E_{∞} ; at pH 2.6 and 4.3 the E_{∞} values for the test samples exceeded those for the controls, whereas the reverse was true at pH 8.9 (see also Convy & Wynn, 1967).

Fig. 1. Fibril formation and dissolution for an untreated collagen solution (a), and for collagen solutions treated with semicarbazide (▲) and NaCl (●) at 24.8°C at pH 2.6 (b), at 34°C at pH 4.3 (c) and at 32.1°C at pH 8.9 (d). The treated solutions were exhaustively dialysed at 4°C against 0.1 M-NaCl, pH 4.2, before fibril formation at 25°C at pH 7, and the fibrils were incubated for 22 h at 25°C before cooling to 4°C.

Table 1. *Effect of semicarbazide on the various collagen fractions involved in fibril formation*

Letters T and C refer to solutions treated with semicarbazide and NaCl respectively. (a) and (b) refer to results calculated from hydroxyproline analyses and final extinction values respectively.

pH of treatment	Temperature of treatment (°C)	Sample letter	Extent of precipitation	Fraction stable at 4°C	Fraction solubilized at 4°C	
			(a)	(a)	(a)	(b)
	Untreated collagen		0.85	0.81	0.04	0.2
2.6	24.8	C	0.91	0.82	0.09	0.13
		T	0.90	0.67	0.23	0.54
4.3	25.6	C	0.89	0.88	0.01	0.05
		T	0.83	0.68	0.15	0.30
	32.0	C	0.83	0.81	0.02	0.06
		T	0.82	0.66	0.16	0.26
	34.0	C	0.85	0.80	0.05	0.07
		T	0.87	0.65	0.22	0.32
	35.2	C	0.83	0.82	0.01	0.06
		T	0.75	0.64	0.11	0.15
	36.1	C	0.77	0.75	0.02	0.07
		T	0.73	0.63	0.10	0.24
8.9	25.7	C	0.93	0.85	0.08	0.06
		T	0.95	0.67	0.28	0.33
	32.1	C	0.88	0.81	0.07	0.12
		T	0.89	0.67	0.21	0.46
	34.2	C	0.89	0.87	0.02	0.16
		T	0.91	0.70	0.21	0.36
	35.4	C	0.89	0.81	0.07	0.13
		T	0.95	0.58	0.37	0.47
	36.9	C	0.85	0.81	0.04	0.15
		T	0.85	0.55	0.30	0.57
	39.8	C	0.87	0.81	0.06	0.14
		T	0.85	0.58	0.27	0.54

Hydroxyproline analyses. Quantitative values for the various fractions involved in fibril formation and dissolution are given in Table 1, from which certain generalizations can be made. The only samples showing a significant decrease in precipitability were those that had been heated above 34°C at pH 4.3. This effect was slightly greater with the semicarbazide-treated samples. The fraction of fibrils that redissolved on cooling was increased after semicarbazide treatment at all pH values and temperatures studied, and confirmed the turbidimetric observations. Semicarbazide decreased the fraction of fibrils that resisted dissolution.

Difference spectroscopy. Fig. 2 shows (a) typical difference spectra observed between solutions treated with semicarbazide and solutions treated with sodium chloride at the same pH and temperature, and (b) the difference spectrum observed between a solution treated with sodium chloride at pH 2.6 and one similarly treated at pH 4.2. The spectra recorded between solutions treated with semicarbazide and sodium chloride under identical conditions exhibited two positive peaks at 220–225nm and 265nm respectively. The peak at 220–225nm was previously correlated with conformational changes resulting from the effects of

both semicarbazide and pH differences (Convy & Wynn, 1967). These difference spectra are essentially different from those observed with solutions treated at differing pH in the absence of semicarbazide (continuous curve in Fig. 2) in that the latter spectrum shows only one peak at 220nm and a gradual decrease in the region 230–310nm which possibly represents additional structural changes. Since there was no difference in the pH of treatment with semicarbazide or sodium chloride, the peak at 265nm must be due only to the effect of semicarbazide. Doyle & Bello (1968) have shown that modification of collagen structure produces only small extinction changes at 265nm even in concentrated solutions. It is unlikely that the large ΔE values observed at 265nm in this experiment are due to such conformational changes and some more specific effect of semicarbazides is indicated. Additional studies (Convy & Wynn, 1966b) indicated a direct binding of semicarbazide to the collagen molecule that, together with the observation that semicarbazone derivatives of $\alpha\beta$ -unsaturated aldehydes absorb maximally at 265nm (Evans & Gillam, 1943), suggested that semicarbazide could bind to similar groups on the collagen molecule. The amounts of semicarbazide (as the

Fig. 2. Difference spectra at room temperature between (a) acid-soluble collagen solutions treated with semicarbazide and similar solutions treated with NaCl at 24.8°C at pH 2.6 (-----), at 34.0°C at pH 4.3 (-·-·-) and at 34.2°C at pH 8.9 (·····), and (b) between a collagen solution treated with NaCl at pH 2.6 and one similarly treated at pH 4.2 at 34.2°C (—). The treated solutions were exhaustively dialysed at 4°C against 0.1 M-NaCl, pH 4.2, before spectroscopy. The reference cell contained NaCl-treated solutions for (a) and the solution treated with NaCl at pH 4.2 for (b).

semicarbazone derivative) bound to collagen under various conditions were therefore calculated from (a) collagen concentration and (b) the peak height at 265 nm, measured by the difference in maximum extinction difference at 265 nm and the minimum extinction difference at 310 nm. The semicarbazone derivative was assumed to have a molar extinction coefficient of 2×10^4 (Evans & Gillam, 1943). Table 2 indicates that more semicarbazide was bound after treatment at pH 2.6 and 4.3 than was bound after treatment at pH 8.9.

DISCUSSION

Wood & Keech (1960) showed that for a given precipitability, any variation in E_∞ with experimental conditions was due to a change in the structure of the precipitated fibrils, E_∞ increasing as the final fibril width increased. The observed differences in E_∞ after semicarbazide treatment at the various pH values (Figs. 1b, 1c and 1d) cannot be attributed to differences in the extent of precipitation as was previously proposed (Convy & Wynn, 1967), since, except for solutions heated above 34°C at pH 4.3, the extent of precipitation was approximately independent of pH, temperature and semicarbazide treatment (Table 1). The increased and decreased values of the final extinction before cooling must therefore reflect an increase and a decrease respectively in the final fibril width.

Table 2. *Effect of pH and temperature on the binding of semicarbazide to the $\alpha\beta$ -unsaturated aldehydes of the collagen molecule*

$\Delta E_{\max. (265\text{nm})} - \Delta E_{\min. (310\text{nm})}$ represents the height of the peak at 265 nm observed in the u.v. difference spectra between test and control solutions. The concentration of the semicarbazone derivative was calculated from the peak height assuming $\epsilon 2 \times 10^4$. The molecular weight of collagen was assumed to be 3×10^5 .

pH of treatment	Temperature of treatment (°C)	$\Delta E_{\max. (265\text{nm})} - \Delta E_{\min. (310\text{nm})}$	Semicarbazone derivative/collagen molar ratio
2.6	24.8	0.072	0.90
	32.0	0.045	0.86
	34.0	0.050	0.95
	36.3	0.035	0.67
4.3	25.5	0.070	1.10
	32.7	0.065	0.83
	34.0	0.065	0.98
	35.3	0.045	0.68
	36.2	0.055	0.83
	38.3	0.045	0.68
8.9	25.7	0.030	0.53
	32.1	0.028	0.49
	34.2	0.045	0.80
	35.4	0.020	0.35
	36.5	0.016	0.28
	39.8	0.027	0.47

Difference spectroscopy indicated the binding of semicarbazide to an $\alpha\beta$ -unsaturated aldehyde on the collagen molecule. Since an $\alpha\beta$ -unsaturated aldehyde is believed to be the main intramolecular cross-link of the β -unit (Bornstein, Kang & Piez, 1966), it is concluded that semicarbazide is binding to this cross-link. The observation that greater amounts of semicarbazide were bound after treatment at pH 2.6 and 4.3 (where E_∞ increased) than at pH 8.9 (where E_∞ decreased) suggested that the amount of bound semicarbazide might correlate with E_∞ and final fibril width.

The increased extent of fibril dissolution after semicarbazide treatment (Figs. 1b, 1c and 1d, and Table 1) indicated that semicarbazide decreased fibril stability as a result of the inhibition of covalent intermolecular cross-linking. The fact that both intra- and inter-molecular cross-links involve aldehyde functions (Bailey, 1968; Tanzer, 1967; Deshmukh & Nimni, 1969) suggests that the binding of semicarbazide to the aldehyde groups present in the intramolecular cross-link of the collagen molecules in solution inhibits the formation of aldehyde-mediated intermolecular cross-links between the individual molecules in the reconstituted fibrils, and consequently decreases fibril stability.

The collagen fraction that resisted dissolution at 4°C, which, allowing for a certain degree of stabilization during incubation at 25°C, may be equated with nucleus-forming collagen (Fessler, 1960a,b; Wood, 1962), was decreased after semicarbazide treatment (Table 1). Since Wood & Keech (1960) showed that E_∞ (and final fibril width) was determined during the formation of this fraction, differences in E_∞ after semicarbazide treatment might be due to the action of semicarbazide on this specific collagen fraction. It is noteworthy that nucleus-forming collagen is characterized by a high content of β -units, which contain the potential binding site for semicarbazide.

Recent research has shown that the quantities of (a) α - and β -units (Piez, Lewis, Martin & Gross, 1961; Piez, 1964, 1965) and (b) aldehydes (Piez, Martin, Kang & Bornstein, 1966) vary with the source and preparation of soluble collagen. The effect of lathyrogens *in vitro* will consequently vary considerably with the type of soluble collagen studied. Wood (1963) observed no marked differences in the initial stability between fibrils reconstituted from semicarbazide-treated and control solutions of neutral-salt-soluble collagen. Since this collagen contains few β -units and hence few $\alpha\beta$ -unsaturated aldehydes, the amount of bound semicarbazide would be small and intermolecular cross-linking (hence fibril stability) would not be significantly altered. On the other hand, Tanzer, Monroe & Gross (1966) studied the effect of thio-

semicarbazide on acid-soluble collagen and observed results similar to those reported here.

It is concluded that the binding of semicarbazide to the intramolecular cross-link of collagen molecules in solution affects (a) the nature (final fibril width) and (b) the stability of fibrils reconstituted from solution. The significance of these results *in vivo* is not immediately apparent, although a change in cellular pH in the presence of a lathyrogenic-like metabolite could change the nature and stability of the fibrils formed and impair the normal function of collagenous tissues.

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