The Action of Semicarbazide on the Aggregation of the Tropocollagen Macromolecule

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1. A difference in conformation was found between the collagen in solutions treated with semicarbazide hydrochloride and those treated with sodium chloride. This difference could be correlated with the difference in extent of aggregation between the fibrils precipitated from these solutions. 2. The action of semicarbazide hydrochloride depended on the pH and temperature of treatment in a complex manner. At constant temperature semicarbazide enhanced aggregation at pH values less than 4.3, but decreased aggregation was observed at pH values greater than 5.0. At pH 4.3 the effect of semicarbazide on aggregation varied with temperature, the tendency to increased aggregation being more pronounced at 34° and 36–37°. Similar increased aggregation tendencies superimposed on an overall decreased aggregation were observed at these temperatures at pH 8.9. 3. A specific binding of semicarbazide to the collagen molecule was indicated.

Soluble collagens may be extracted from collagenous tissues in a variety of ways, which include the use of cold neutral salt solutions or organic acids (Jackson, 1957). Typical collagen fibrils may be reconstituted *in vitro* from the soluble collagen solutions by altering certain experimental conditions including temperature and pH. It is possible that a study of this reconstitution may provide some insight into the mechanism of fibrillogenesis *in vivo*. Fibril formation *in vitro* is considered to be the result of two consecutive processes: (a) the formation of nuclei by the aggregation of soluble collagen particles, and (b) the growth of these nuclei by the accretion of further soluble collagen (Wood, 1960). The shape of the precipitation curve, which may be followed turbidimetrically at 400 m μ , consists of a lag period, during which no fibrils are observed, followed by a sigmoid portion that constitutes the growth phase, the final extinction value (E_{∞}) denoting complete precipitation.

The fibrils precipitated from collagen solutions are composed of a basic structural unit, the tropocollagen macromolecule, which exists in solution as a triple helix (γ -unit) composed of three polypeptide chains (α -sub-units). The most probable arrangement of the α -chains would appear to be the collagen II model proposed by Rich & Crick (1961). It is further believed (Grassmann, Hannig & Engel, 1961) that the predominant species in solutions of acid-soluble collagen is one in which two of the three α -chains are covalently bonded to form a β -sub-unit, whereas in solutions of collagen soluble in

neutral salt the predominant species is one in which there is no covalent cross-linking.

When solutions of acid-soluble collagen are heated above 32°, the specific viscosity and optical laevorotation decrease, a 50° decrease being found at the denaturation temperature (Doty & Nishihara, 1958). Denaturation is a two-stage process (Engel, 1962); the rod-like structure of the triple helix collapses and this is followed by the separation of the polypeptide chains into α - and β -sub-units. In addition, a small amount of the γ -form (with the three chains covalently linked) remains in the denatured solution. The extent of denaturation depends on many factors, including temperature, pH, time, ionic strength and the presence of other substances, e.g. urea (Doty & Nishihara, 1958; Kühn, Engel, Zimmermann & Grassmann, 1964). The denaturation process is reversible, and much attention has focused on the re-formation and stabilization of the collagen fold from completely denatured collagen (gelatin) solutions after quenching to a temperature below the denaturation temperature. The mode and extent of renaturation depends on the composition of α -, β - and γ -sub-units of the gelatin solution, the temperature of renaturation and the time allowed for renaturation. In this respect, the rate of renaturation is more rapid for gelatins containing multi-chain β - and γ -components than for those containing α -chains (Altgelt, Hodge & Schmitt, 1961; Engel, 1962; Drake & Veis, 1962). Renaturation is also aided by the presence of covalent cross-links (Drake & Veis, 1964).

Much research has focused on the relationship

between this basic tropocollagen unit and the native collagen fibre, which in the electron microscope shows a pattern of dark and light transverse bands repeating at approx. 640 Å (Gross, Highberger & Schmitt, 1955). This pattern is an expression of the way in which tropocollagen units are aggregated laterally.

Tropocollagen can aggregate *in vitro* to form fibrils with periodicities other than the native 640 Å type. Thus a periodicity of 210 Å is found when collagen is precipitated from a 2% sodium chloride solution, and precipitation from solutions of 0.1% acid glycoprotein or 0.05% ATP produces fibrils with a 2600 Å periodicity, named respectively the fibrous long-spacing and segment long-spacing forms. All these various forms of aggregates are interconvertible (Gross, 1956) and various models have been proposed to account for these different aggregates of tropocollagen molecules with specific periodicities. Hodge & Schmitt (1960) suggested that in the native (640 Å) fibril the tropocollagen units were aggregated laterally with each unit staggered by a quarter of its length. Kühn & Zimmer (1961) have indicated that the macromolecules in the native and fibrous long-spacing forms are aggregated by end overlap, and Schmitt & Hodge (1960) have suggested end-to-end aggregation by a coiling around each other of the polypeptide tails, which project from the ends of the tropocollagen units.

Grant, Horne & Cox (1965) have suggested that there is no need to postulate any of these modes of end aggregation. They propose a model in which the tropocollagen molecule consists of alternating bonding and non-bonding regions. Correct juxtaposition of two such bonding (polar) regions results in strong lateral attraction and hence aggregation, and the repeat period depends on the length and type of each bonding zone. Thus no model accounts unequivocally for the various modes of aggregation and the relationship between the basic tropocollagen unit and the fibrils composed of these units.

Wood & Keech (1960) showed that fibrils precipitated from acid-soluble collagen solutions under their conditions had a periodicity of 640 Å. Wood (1963a) extended this work by a study of the effect of semicarbazide (a lathyrogen) on the aggregation of neutral-salt-soluble collagen and on the subsequent maturation of the fibrils formed from these solutions. His results confirmed the previous hypothesis that lathyrogens act by altering the aggregation stage of collagen fibrillogenesis, possibly by inhibiting the cross-linking mechanism (Martin, Piez & Lewis, 1963).

In the present work, part of which has been reported in a preliminary communication (Convy & Wynn, 1966), the action of semicarbazide on the formation *in vitro* of fibrils from acetic acid-soluble

collagen solutions was studied under various experimental conditions.

MATERIALS AND METHODS

Acid-soluble collagen solutions. Purified calf dermis, which had previously been exhaustively extracted with neutral-salt solutions and freeze-dried, was extracted for 18 hr. with 0.5N-acetic acid at 4° and the resultant acid-soluble collagen solution purified by the method of Wood & Keech (1960).

Chemical. AnalaR semicarbazide hydrochloride (British Drug Houses Ltd., Poole, Dorset) was used.

Determination of collagen concentration. Acid-soluble collagen solutions were hydrolysed in 6N-HCl for 18 hr. at 105° and the free hydroxyproline liberated was estimated by the method of Woessner (1961). The concentration of collagen in the original solution was calculated assuming that collagen contains 13.6% (w/w) of hydroxyproline. The concentration of solutions of acid-soluble collagen extracted from calf dermis in the present investigation varied between 0.05 and 0.1% (w/v) and was less than that reported by Wood & Keech (1960).

Treatment of collagen with semicarbazide. Solutions of acid-soluble collagen in 0.1M-NaCl containing 5mM-sodium acetate adjusted to pH 4.2 with 5mN-acetic acid were treated with an excess of semicarbazide hydrochloride for 30 min. at temperatures ranging from 25° to 40°. The pH of the final incubation mixture was 2.7, the final collagen concentration varying between 0.03 and 0.07% but being constant for each series of experiments, and the final molar concentration ratio of collagen to semicarbazide was approx. 1:20000. Controls substituting NaCl for semicarbazide hydrochloride were similarly treated at pH 4.2.

To allow for the pH discrepancy in this system, control experiments were performed in which acid-soluble collagen solutions at pH 4.2 were adjusted to a final pH of 2.7 by the addition of a suitable volume of 7mN-HCl, and subsequently treated as above. Care was taken to ensure that the collagen concentrations of the NaCl and semicarbazide or HCl reaction mixtures were the same.

The action of semicarbazide hydrochloride at pH 4.3 and 8.9 over a range of temperatures was studied by the addition of solutions of semicarbazide in either 0.2M-sodium acetate adjusted to pH 5.9 with 0.2N-acetic acid, or 0.1M-tris adjusted to pH 9.6 with 0.2N-NaOH, to acid-soluble collagen solutions, pH 4.2. The pH values of the final incubation mixtures were 4.3 and 8.9 respectively. As controls, acid-soluble collagen solutions, pH 4.2, were treated with solutions of NaCl in either 0.2M-sodium acetate adjusted to pH 4.5 with 0.2N-acetic acid or 0.1M-tris adjusted to pH 9.2 with 0.1N-HCl. The final pH values of the control mixtures were again 4.3 and 8.9.

The design of experiments to study the effect of semicarbazide hydrochloride on acid-soluble collagen at constant temperature but various pH values was more difficult because of the highly acidic nature of the semicarbazide hydrochloride. Two different approaches to the problem were tried. First, a solution of semicarbazide hydrochloride was added to an acid-soluble collagen solution at pH 4.2 and the final pH was adjusted with 0.2N-NaOH. Mixtures of acid-soluble collagen and NaCl were similarly adjusted. In this way it was possible to prepare reaction mixtures from pH 5.0 to 10.5 without the introduction of other anions that

might possibly affect the final result. However, two serious disadvantages of this technique were the lack of buffer capacity and the irreproducibility of the results. In the second method, mixtures of equal volumes of a semicarbazide hydrochloride solution, adjusted to pH 7.2 with 0.2N-NaOH, and 0.2M- Na_2HPO_4 adjusted to various suitable pH values with 0.1M-citric acid, were added to acid-soluble collagen solutions, pH 4.2, to give final pH values ranging from 4.2 to 7.4. A mixture of equal volumes of semicarbazide hydrochloride solution, adjusted to pH 7.2 with 0.2N-NaOH, and 0.1N-tris adjusted to pH 9.8 with 0.1N-HCl was used to give a pH of 8.6. This method has the benefit of greater buffering power and reproducibility, but introduces additional anions in phosphate and citrate. The results of experiments performed in the two ways were therefore compared. Treatment at the various pH values was carried out for 30 min. at 32.5° or 32.2°.

At the end of the 30 min. period under the various conditions, the test and control solutions were exhaustively dialysed at 4° against 0.1M-NaCl containing 5mM-sodium acetate adjusted to pH 4.2 with 5mM-acetic acid.

Turbidimetric study of fibril formation. The formation of fibrils from portions of the non-diffusible material of the test and control solutions was followed turbidimetrically at 25° by the method of Wood & Keech (1960), precipitation being initiated by the addition of 74mM- KH_2PO_4 adjusted to pH 6.8–6.9 with 74mM-NaOH and NaCl added to a final ionic strength of 0.21.

During the investigation two spectrophotometers, each fitted with a constant-temperature cell housing, were used to follow the increase in extinction at 400m μ : the Unicam SP.600 spectrophotometer and the Optica CF4R recording spectrophotometer. The Unicam instrument gave lower extinction values than the Optica, owing to the greater light-scattering effect in the former spectrophotometer. The final extinction values, corresponding to the extent of precipitation, for the test $E_{\infty(T)}$ and control $E_{\infty(C)}$ solutions were noted, suitable extinction ranges being chosen so that it was possible to determine the extinction to ± 0.01 .

Ultraviolet difference spectra. Difference spectra between the non-diffusible material of the test and control solutions treated under the various conditions were recorded directly at room temperature with the Optica CF4R spectrophotometer. The difference spectra in the range 210–310m μ were recorded relative to a base line obtained by adjusting the instrument to 50% transmission with water in the reference and test cells.

These curves were reproducible, and the error in estimating ΔE was between 2 and 4% depending on the absolute value of ΔE .

RESULTS

Effect of temperature. A typical pattern of fibril formation from acid-soluble collagen solutions treated with semicarbazide and sodium chloride at pH 2.7 and 4.2 respectively at 32.1° is illustrated in Fig. 1. The difference ($E_{\infty(T)} - E_{\infty(C)}$) for a range of temperatures is shown in Table 1. The final extinction value of the control solutions $E_{\infty(C)}$ did not vary significantly with treatment at temperatures below the denaturation temperature (36–37°). However, the final extinction value

Fig. 1. Fibril formation from acid-soluble collagen solutions treated with semicarbazide hydrochloride at 32.1° at pH 2.7 (○), and with NaCl at pH 4.2 (●), and exhaustively dialysed against 0.1M-NaCl, pH 4.2. The conditions for precipitation required: temperature 25°, I , 0.21 and pH 6.9.

Fig. 2. Difference spectrum at room temperature between acid-soluble collagen solutions treated at 34.1° with semicarbazide hydrochloride at pH 2.7 and NaCl at pH 4.2, and exhaustively dialysed against 0.1M-NaCl, pH 4.2. The reference cell contained NaCl-treated solutions.

reached in the test solutions $E_{\infty(T)}$ was markedly decreased after treatment above 32.1°.

The ultraviolet difference spectrum between the test and control solutions treated at 34.1° is shown in Fig. 2. A positive peak was observed and its height was measured by the difference between the maximum extinction difference that was observed at 220m μ and the minimum extinction difference that was observed at 230–245m μ ($\Delta E_{\text{max. (220 m}\mu)} - \Delta E_{\text{min. (230-245 m}\mu)}$). The variation of this peak height with temperature is shown in Table 1. The peak height was small after treatment below 32.4°, rose to a maximum at 34.1° and thereafter decreased slowly with a shoulder at 36.6°.

Fig. 3 shows that the graphs of peak height and ($E_{\infty(T)} - E_{\infty(C)}$) plotted against temperature are

Table 1. *Fibril formation and spectral differences of collagen treated with semicarbazide hydrochloride at various temperatures*

E_{∞} is the final extinction value at 440m μ recorded on the Unicam SP.600 (series a) and the Optica CF4R (series b) spectrophotometers during fibril formation (see the Materials and Methods section). Subscripts (T) and (C) refer to solutions treated with semicarbazide at pH 2.7 (tests) and with NaCl at pH 4.2 (controls) respectively. ($\Delta E_{\text{max. (220 m}\mu\text{)}} - \Delta E_{\text{min. (230-245 m}\mu\text{)}}$) represents the height of the peak observed in the ultraviolet difference spectra between the test and control solutions recorded on the Optica CF4R spectrophotometer.

(a)				(b)				$(\Delta E_{\text{max. (220 m}\mu\text{)}} - \Delta E_{\text{min. (230-245 m}\mu\text{)}})$	
Temp.	$E_{\infty (T)}$	$E_{\infty (C)}$	$(E_{\infty (T)} - E_{\infty (C)})$	Temp.	$E_{\infty (T)}$	$E_{\infty (C)}$	$(E_{\infty (T)} - E_{\infty (C)})$	Temp.	$\Delta E_{\text{max. (220 m}\mu\text{)}} - \Delta E_{\text{min. (230-245 m}\mu\text{)}}$
26.7°	1.34	1.18	+0.16	27.7°	1.50	1.40	+0.10	28.0°	+0.050
32.1	1.14	1.26	-0.12	32.7	0.29	1.36	-1.07	32.4	+0.040
34.9	0.37	1.18	-0.81	34.0	0.09	1.31	-1.22	34.1	+0.170
35.8	0.37	1.16	-0.79	35.4	0.01	1.10	-1.09	35.7	+0.145
36.6	0.32	1.09	-0.77	36.7	0.02	1.19	-1.17	36.6	+0.140
39.4	0.23	0.17	+0.06	40.2	0.008	0.08	-0.072	40.4	+0.020

Fig. 3. Correlation between fibril formation and spectral differences of collagen treated with semicarbazide at various temperatures. ●, Peak height of ultraviolet difference spectrum ($\Delta E_{\text{max. (220 m}\mu\text{)}} - \Delta E_{\text{min. (230-245 m}\mu\text{)}}$) between semicarbazide- and NaCl-treated collagen solutions, treated at pH 2.7 and 4.2 respectively. ■, Difference in final extinctions of precipitates from solutions treated with semicarbazide at pH 2.7 and NaCl at pH 4.2 ($E_{\infty (T)} - E_{\infty (C)}$); measured on the Unicam SP.600 instrument. ○, As ■, but measured on the Optica CF4R spectrophotometer.

Fig. 4. Correlation between fibril formation and spectral differences of collagen treated with HCl at various temperatures. ●, Peak height of ultraviolet difference spectrum ($\Delta E_{\text{max. (220 m}\mu\text{)}} - \Delta E_{\text{min. (230-245 m}\mu\text{)}}$) between HCl- and NaCl-treated collagen solutions, treated at pH 2.7 and 4.2 respectively. ○, Difference in final extinctions of precipitates from solutions treated with HCl at pH 2.7 and NaCl at pH 4.2 ($E_{\infty (T)} - E_{\infty (C)}$); measured on the Optica CF4R instrument.

remarkably similar; the upper graph possesses a maximum at 34.1° and a shoulder at 36.6° that correspond to the minimum and shoulder shown in the lower graphs.

Fig. 4 illustrates the comparable values of peak height obtained from ultraviolet difference spectroscopy, and ($E_{\infty (T)} - E_{\infty (C)}$) obtained from fibril formation, plotted against the reaction temperature for acid-soluble collagen solutions treated with hydrochloric acid at pH 2.7 to compensate for the pH of the semicarbazide mixture. Once again a correlation between peak height and final extinction difference was found, but the inflexion at 34.0° had disappeared. It would appear that this inflexion observed at 34.0° in Fig. 3 is predominantly due to the effect of semicarbazide, and the shoulder at

Table 2. Fibril formation from collagen solutions treated with semicarbazide hydrochloride at various pH values and temperatures

$E_{\infty(T)}$ and $E_{\infty(C)}$ are as described in Table 1. Series (i) and (ii) refer to solutions adjusted to the various pH values by methods 1 and 2 respectively (see the Materials and Methods section).

	pH	Temp.	$E_{\infty(T)}$	$E_{\infty(C)}$	$(E_{\infty(T)} - E_{\infty(C)})$
	4.3	25.5°	2.18	1.50	+0.68
	4.3	32.7	2.18	1.83	+0.35
	4.3	34.0	2.30	1.92	+0.38
	4.3	35.3	2.27	1.98	+0.25
	4.3	36.2	2.07	1.75	+0.32
	4.3	38.3	0.00	0.00	0.00
	8.9	28.7	0.95	1.26	-0.31
	8.9	31.9	0.76	1.32	-0.56
	8.9	34.1	0.78	1.31	-0.53
	8.9	35.7	0.84	1.44	-0.60
	8.9	37.1	0.94	1.35	-0.41
	8.9	39.6	0.80	1.52	-0.72
	(i) 5.05	32.2	1.03	1.60	-0.57
	6.15	32.2	0.86	1.15	-0.29
	9.65	32.2	0.22	1.24	-1.02
(ii)	10.50	32.2	0.34	1.09	-0.75
	4.23	32.5	1.92	1.79	+0.13
	4.98	32.5	1.59	1.82	-0.23
	5.84	32.5	1.37	1.46	-0.09
	6.83	32.5	1.17	1.33	-0.16
	7.40	32.5	0.90	1.70	-0.80
	8.60	32.5	1.25	1.54	-0.29

36.6° (approximately the denaturation temperature) is primarily due to the difference in the pH (i.e. 2.7 and 4.2) at which test and control solutions were treated. $E_{\infty(T)}$ was significantly less marked at all temperatures in solutions treated with hydrochloric acid than with semicarbazide at pH 2.7, suggesting a possible enhancement of aggregation by semicarbazide at this pH.

Effect of pH. The values of $E_{\infty(T)}$, $E_{\infty(C)}$ and $(E_{\infty(T)} - E_{\infty(C)})$ for fibril formation from solutions treated with semicarbazide at pH 4.3 and 8.9 at various temperatures, and over the pH range 4.2–10.5 at 32.5° or 32.2°, are shown in Table 2. The value of $(E_{\infty(T)} - E_{\infty(C)})$ for solutions treated at pH 4.3 at all temperatures below 37° is positive, whereas this value is negative for solutions treated below 40° at pH 8.9. The final extinction difference was also positive after treatment at pH 4.2 and 32.5°. Above pH 5.0, the final extinction difference was negative irrespective of the method of pH adjustment (see the Materials and Methods section).

The variation of $(E_{\infty(T)} - E_{\infty(C)})$ and peak height at pH 4.3 with temperature is shown in Fig. 5. Both quantities varied similarly with temperature and two maxima were observed at 34.0° and 36.2°.

Fig. 5. Correlation between fibril formation and spectral differences of collagen treated with semicarbazide at pH 4.3 at various temperatures. ●, Peak height of ultraviolet difference spectrum $(\Delta E_{\max. (220 \text{ m}\mu)} - \Delta E_{\min. (230-245 \text{ m}\mu)})$ between semicarbazide- and NaCl-treated collagen solutions both treated at pH 4.3. ○, Difference in final extinctions of precipitates from solutions treated with semicarbazide and NaCl at pH 4.3. $(E_{\infty(T)} - E_{\infty(C)})$; measured on the Optica CF4R instrument.

semicarbazide, as the pH of both tests and controls is the same in this experiment. Ultraviolet difference spectra were not obtained for solutions treated at other pH values, but the curve resulting from a plot of $(E_{\infty(T)} - E_{\infty(C)})$ against temperature after treatment at pH 8.9 once again showed two maxima at 34.1° and 37.1°, indicating that decreased aggregation observed at all temperatures after treatment at pH 8.9 was less extensive after treatment at these two temperatures [i.e. $(E_{\infty(T)} - E_{\infty(C)})$ was less negative].

DISCUSSION

In the present study, ultraviolet difference spectroscopy was used to detect conformational changes in the collagen molecules after treatment and renaturation. Spectral changes in the 220–240 mμ region have been attributed to an effect of main-chain conformational changes on peptide-group absorption, but it is possible that other phenomena also contribute to changes in this region (Glazer & Smith, 1960, 1961). Wood (1963b) observed a positive peak at 223 mμ in the difference spectrum between denatured and native acid-soluble collagen solutions.

In addition to indicating a possible mechanism of fibrillogenesis *in vivo*, fibril formation *in vitro* is one of the criteria of renaturation. It follows that the degree of the conformational changes occurring in

the tropocollagen molecules, and the extent of fibril formation, will depend on the conditions used in the treatment of acid-soluble collagen solutions and the conditions under which renaturation occurs. In the present study the conditions for renaturation were constant, and the observations noted during fibril formation and in the ultraviolet difference spectra from renatured acid-soluble collagen solutions reflect the effect of the various experimental conditions on the tropocollagen molecule.

Fig. 4 illustrates the difference in conformation (measured by the peak height of the ultraviolet difference spectra) between the molecules in solutions treated at pH 2.7 and 4.2, at a range of temperatures in the absence of semicarbazide. The difference was a maximum after treatment at 36.9°, the temperature at which the difference in the extent of aggregation ($E_{\infty(T)} - E_{\infty(C)}$) was a minimum, indicating that the conformational changes caused by the difference in pH were manifested in an effect on fibril formation. Such a correlation between conformational changes and the difference in extent of aggregation was also found when acid-soluble collagen solutions were treated with semicarbazide at pH 2.7 relative to the controls at pH 4.2 (Fig. 3). However, in this case an inflexion was observed at 34° in addition to the effect at 36.6° (shown by the shoulder). It thus appears that the effect of semicarbazide alone obtains at 34°, and the effect observed at 36.6° is probably due to the sum of the effects of the pH difference and semicarbazide.

At pH 4.3 (Fig. 5 and Table 2) it was shown that the action of semicarbazide indeed exerted a maximum effect at two temperatures, 34° and 36.2° respectively. Since the test and control solutions were treated at the same pH and temperature, both maxima must be due to the semicarbazide effect. If semicarbazide simply lowered the denaturation temperature no maximum would be observed at 36.2°. It therefore appears that the conformation of the molecules in solution during treatment at 34° and 36.2° is more favourable to the action of semicarbazide than at intermediate temperatures, and that after renaturation the difference in aggregation of the fibrils precipitated from solutions treated at these two temperatures, with and without semicarbazide, is increased. Similar behaviour was noted at pH 8.9 (Table 2). Semicarbazide appears to have induced a change in the structure of the collagen molecule in addition to the conformational change produced by heat alone.

The effect of semicarbazide at a range of pH values at a particular temperature is even more complex (Table 2). Treatment with semicarbazide below pH 4.2 resulted in increased aggregation relative to the control solution [$(E_{\infty(T)} - E_{\infty(C)})$ was positive], but treatment above pH 5.0 resulted in decreased aggregation [$(E_{\infty(T)} - E_{\infty(C)})$ was nega-

tive]. In this respect Davison & Drake (1966) have suggested that one or more specifically located carboxyl groups that enhance fibrogenesis have a pK in the range 4.2–4.5. Fibril formation occurs in collagen solutions in the absence of semicarbazide when the pH is raised from 4.2 to 4.5. This is the region in which the sign of $(E_{\infty(T)} - E_{\infty(C)})$ is reversed. It is possible that the site of action of semicarbazide is located near the specific carboxyl groups that enhance aggregation, and that the effects of pH observed with test and control collagen solutions are not completely independent. Other explanations are, however, possible: Wood (1962) showed that solutions of neutral-salt-soluble collagen (and possibly acid-soluble collagen) were heterogeneous with respect to the ability of their constituent collagen molecules to aggregate to form fibrils, and suggested that this fibril-forming ability was related to the sub-unit composition of the collagen molecules. Solutions of neutral-salt-soluble collagen could be divided into three fractions, A, B and C. Fraction C was designated the nucleus-forming collagen (responsible for the lag phase) and was characterized by a high proportion of the β -component relative to the unfractionated material. Fraction A, the growth collagen, was responsible for the growth phase and was similar to the unfractionated material in its β -sub-unit composition. If the same heterogeneity exists in solutions of acid-soluble collagen, which contain a higher β/α ratio in the unfractionated material than does neutral-salt-soluble collagen, it is possible that semicarbazide affects the β/α ratio in fraction C, and that this effect is pH-dependent. (This hypothesis could also explain the effect of semicarbazide at various temperatures at a constant pH.) On the other hand, during treatment above pH 5.0 (but not below pH 4.2) collagen is precipitated from solution, and, although the fibrils redissolve on dialysis, the nuclei that are irreversibly formed remain for the accretion of growth collagen during the subsequent fibril formation *in vitro*. Semicarbazide could obviously affect collagen fibrils in quite a different way from the constituent tropocollagen molecules in solution.

Wood (1963a) treated neutral-salt-soluble collagen solutions with semicarbazide at pH 7.0 and 35° and studied fibril formation subsequent to dialysis. He observed an increased lag phase after semicarbazide treatment, but the final extent of precipitation was approximately the same for the test and control solutions. We observed no large increase in the lag phase after treatment at this pH, but observed a decreased final extinction value relative to the control solution, as was also shown at all pH values above pH 5.0. Since acid-soluble collagen solutions contain a higher proportion of the β -component and fraction C than neutral-salt-soluble collagen, this difference may account for the difference in

action of semicarbazide on acid-soluble and neutral-salt-soluble collagens.

In a preliminary communication (Convy & Wynn, 1966), it was noted that at pH 2.7 and 37° acid-soluble collagen solutions incorporated approx. 6.0 moles of semicarbazide/mole of tropocollagen (2 moles/ α -unit). Moreover, further studies (S. Convy & C. H. Wynn, unpublished work) indicate that semicarbazide actually binds covalently to the ester bonds proposed by Gallop (1964), and the extent of this binding varies according to the pH and temperature. It is possible that since the ester bonds are found in the polar regions of the tropocollagen molecule, which according to Grant *et al.* (1965) are the bonding zones, the attack on the ester bonds could lead to an alteration of the number and type of bonding zones interacting and therefore produce different modes of aggregation. No electron-microscope observations are yet available for the appearance of fibrils treated with semicarbazide under the various conditions, but the fibrils precipitated from the control solutions were presumably of the native type (640 Å), since the conditions of precipitation were those employed by Wood & Keech (1960). If an actual cleavage of the ester bonds is involved, this cleavage cannot be very extensive or no fibrils could be formed even after renaturation is allowed to occur.

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