# Collagen fibril formation in the presence of sodium dodecyl sulphate

George W. DOMBI* and H. Brian HALSALL
*Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221, U.S.A.*

Sodium dodecyl sulphate (SDS) was used to weaken both the electrostatic and the hydrophobic interactions during collagen fibrillogenesis *in vitro*. The rate and extent of fibril formation as well as fibril morphology were affected by SDS concentration. Both the formation of large fibrils at 0.3 mM-SDS and the complete cessation of fibril formation at 0.5 mM-SDS were considered to be the result of SDS-induced conformational changes in the non-helical telopeptides. A possible mechanism of SDS interaction with the *N*-terminal and the distal region of the *C*-terminal telopeptides is offered.

Type I collagen is the major structural protein of the body. Found in the form of fibrillar aggregates, type I collagen gives support and mechanical stability to the connective-tissue matrix where it resides. Cells embedded in the tissue matrix secrete the collagen molecules that in turn form fibrils locally in the presence of sulphate-containing proteoglycans and other matrix-element constituents. The role of the matrix elements in fibril formation is not well understood, but it has been suggested that they may regulate the extent of fibril formation, since fibril width is tissue-specific (Trelstad *et al.*, 1982).

Many studies of fibril formation *in vitro* have been conducted with purified solutions of collagen (Gross & Kirk, 1958; Bensusan, 1960; Wood & Keech, 1960; Wood, 1960; Comper & Veis, 1977). Under appropriate conditions of pH, ionic strength, temperature and collagen concentration, fibrils can be formed that appear identical with those observed *in vivo* (Trelstad *et al.*, 1976; Williams *et al.*, 1978). Because of these studies, it is accepted that fibril formation is based solely on the primary structure of collagen and not on the presence of the other matrix components. It is also generally accepted that the non-helical telopeptides play an important role in the nucleation and growth of fibrils (Hayashi & Nagai, 1974; Comper & Veis, 1977; Helseth *et al.*, 1979; Gelman *et al.*, 1979b; Helseth & Veis, 1981; Capaldi & Chapman, 1982, 1984). These studies suggest that

unique conformations in the telopeptides must be achieved in order for fibrillar events to proceed. It seems clear that the *N*-terminal and the distal region of the *C*-terminal telopeptides are important in the formation of the minimal-overlap aggregates that characterize the initial nucleation and linear association. These studies also indicate a major role for the proximal 'hydrophobic cluster' of the *C*-terminal in directing the lateral association of collagen aggregates.

Although not necessary for fibril formation, a possible role for the components of the extracellular matrix is to exert an influence on the extent of fibril formation by interacting with the telopeptides. In the present study, SDS is used as a sulphate containing amphiphile to investigate fibril formation *in vitro*. The effects of SDS on fibril kinetics are monitored by following changes in solution turbidity and fibril morphology.

## Materials and methods

SDS (electrophoretic grade; Bio-Rad Laboratories) was crystallized from boiling 95% (v/v) ethanol. Other chemicals were obtained from standard commercial suppliers and were of analytical grade where possible.

### Isolation of collagen

Acid-soluble type I collagen was prepared from tail tendons of 6-week-old rats by modifying established procedures (Chandrakasan *et al.*, 1976; Williams *et al.*, 1978; Gelman *et al.*, 1979a). All procedures were conducted at 4°C. A complete description is provided elsewhere (Dombi, 1984). Tendons were excised and washed in ice-cold

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Abbreviation used: SDS, sodium dodecyl sulphate.

\* Present address: Biomedical Science Department, General Motors Research Laboratories, Warren, MI 48090, U.S.A.

50mM-Tris/HCl buffer, pH7.2 (at room temperature), containing the proteinase inhibitors 10mM-*N*-ethylmaleimide, 5mM-benzamidine hydrochloride and 1mM-phenylmethanesulphonyl fluoride. Tendons were solubilized overnight at a ratio of 1g of wet tissue per 100ml of 0.5M-acetic acid containing 2μg each of pepstatin A and leupeptin (Sigma Chemical Co.)/ml. Solubilized collagen was salted out at a final concentration of 5% (w/v) NaCl by the dropwise addition of a solution that contained 25% (w/v) NaCl, 0.5M-acetic acid, 2μg of pepstatin A/ml and 2μg of leupeptin/ml. The collagen precipitate was collected by centrifugation at 20000*g* for 45min, then resolubilized overnight in 0.5M-acetic acid and adjusted to a concentration of 1mg/ml with the same solution. High-$M_r$ collagen aggregates were salted out at 3% (w/v) NaCl and removed by centrifugation at 15000*g* for 45min. Small collagen fragments were separated by precipitating the intact collagen at 4% (w/v) NaCl. This final precipitate was dissolved at approx. 0.5mg/ml in 5mM-acetic acid, and then dialysed against 100vol. of 5mM-acetic acid with three diffusate changes over 48h. The dialysed collagen was clarified by centrifugation at 20000*g* for 60min, then adjusted to a final stock concentration of 0.2mg/ml with 5mM-acetic acid and stored at 4°C.

### Determinations of collagen concentration

Specific rotation values $[A]^{22}_{365} = -1331$ (Kanfer, 1977) and $[\alpha]^{20}_{310} = -2300$ degree·ml·$dm^{-1}·g^{-1}$ (Williams *et al.*, 1978) were used to determine collagen concentration in a Cary 60 spectropolarimeter. Readings were taken in a 1cm-pathlength cell at room temperature. Multiple determinations at the two wavelengths were averaged.

### Fibril formation

Native-type fibrils were formed in a control buffer by mixing 1ml of the stock collagen solution with 1ml of a double-strength salt solution at 15°C to give final concentrations of 30M-Tes, 30mM-$Na_2HPO_4$, 0.2M-NaCl, 0.1mg of collagen/ml and 2.5mM-acetic acid, final pH7.0-7.2 (Williams *et al.*, 1978). Sample buffers were of the same composition including 0.04-1.0mM-SDS. Fibril formation was monitored in a Gilford 250 spectrophotometer. Samples were formed by mixing the two solutions just before use. Portions of the samples were loaded in triplicate into a micro-cell at 15°C and rapidly heated to 22, 24 or 26°C. The required temperature was maintained with a thermoelectric regulator–Gilford no. 2527 thermoprogrammer. Turbidity was recorded continuously over a period of 16h by monitoring the absorbance at 350nm. The resultant fibril-formation curves

were analysed by reference to the pseudokinetic parameter $t_{\frac{1}{2}}$, the time to half-maximum turbidity change.

### Collagen tertiary structure

O.r.d. scans were conducted at room temperature in a Cary 60 spectropolarimeter (Blout *et al.*, 1963). Samples contained 30mM-Tes, 30mM-$Na_2HPO_4$, 0.2M-NaCl, 0.1mg of collagen/ml and 0.1-4.0mM-SDS, final pH7.0-7.2. The observed rotation at 220nm was recorded as a function of SDS concentration.

### Electron microscopy

After 16h of fibril formation, 50μl of sample was retrieved and placed on a 400-mesh carbon-coated copper grid for 1-2min. The grid was slowly drained with filter paper, then floated inverted on a 500μl drop of 1% sodium phosphotungstate, pH7.2, for 10min. The grid was removed and air-dried. The preparation was examined with a Philips EM 300 transmission electron microscope, and micrographs were taken at magnifications of ×12500 and ×42000.

## Results

### Fibril formation

The extent of fibril formation at 26°C in the presence of various SDS concentrations is indicated in Fig. 1. Formation buffers containing 0–0.4mM-SDS produced turbidity curves consisting of the characteristic lag, growth and plateau
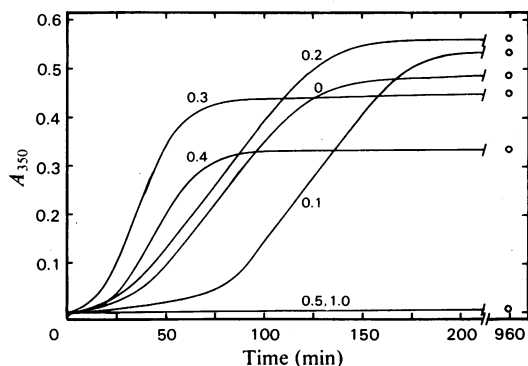


Fig. 1. *Turbidity curves of collagen fibril formation in buffers containing 0–1.0mM-SDS*
Fibril formation was conducted at 26°C by mixing the collagen solution with equal volumes of various formation buffers containing SDS. Cuvette concentrations were 30mM-$Na_2HPO_4$, 200mM-NaCl, 30mM-Tes, 0–1.0mM-SDS, 2.5mM-acetic acid and 0.1mg of collagen/ml, final pH7.0-7.2. SDS concentrations in mM are indicated in the Figure.

phases. Formation buffers containing 0.5 mM- and 1.0 mM-SDS did not produce fibrils. Except with the formation buffer containing 0.1 mM-SDS, all fibrils formed more quickly than the controls. In Fig. 2, $t_{\frac{1}{2}}$ values are plotted against SDS concentration. A general trend is seen; up to 0.4 mM-SDS fibril formation is promoted, and beyond that concentration fibril formation is completely inhibited. The transition appears to be co-operative and complete over a 0.1 mM range.

The effect of temperature on fibril formation was studied in the formation buffer, which contained 0.3 mM-SDS, and in the control buffer at 22, 24 and 26°C. An Arrhenius plot was constructed (Fig. 3) with the kinetic constant expressed as the reciprocal of the $t_{\frac{1}{2}}$ value divided by the molarity of the collagen solution (Sauk *et al.*, 1982). An $M_r$ value of 300000 was used for type I collagen. There is little or no difference between the control and SDS-containing sample. The calculated activation energies for the fibrillar processes were estimated to be $224 \pm 25$ kJ/mol ($54 \pm 6$ kcal/mol) for the control and $204 \pm 17$ kJ/mol ($49 \pm 4$ kcal/mol) for the SDS-containing sample.
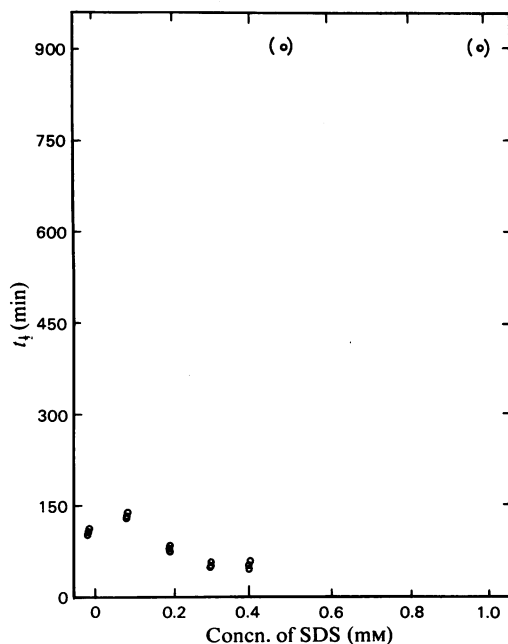
### Effects of SDS on collagen tertiary structure

Fig. 4 indicates the change in the specific rotation measured at 220 nm. Across the range 0–4.0 mM-SDS the change in collagen tertiary structure is 50%. This trend is not linear since rotation
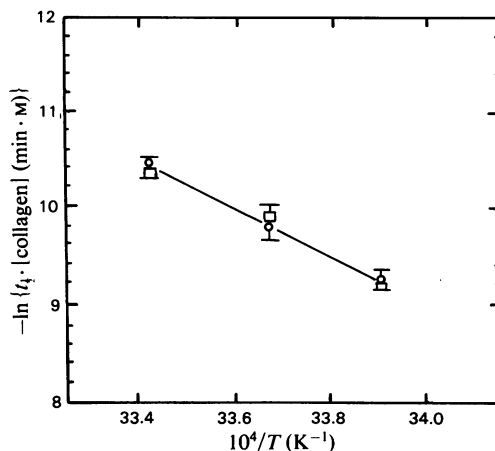


Fig. 3. *Temperature effects on the rate of collagen fibril formation at 22, 24 and 26°C*

The determination of the molar collagen concentration was based on an $M_r$ value at 300000. Control buffer (○) contained 30 mM-Tes, 200 mM-NaCl and 0.1 mg of collagen/ml, final pH 7.0–7.2, and the activation energy was $224.2 \pm 25.1$ kJ/mol (mean $\pm$ s.D.). The sample buffer (□) was of the same composition as the control buffer plus 0.3 mM-SDS, and the activation energy was $204.1 \pm 16.7$ kJ/mol (mean $\pm$ s.D.).
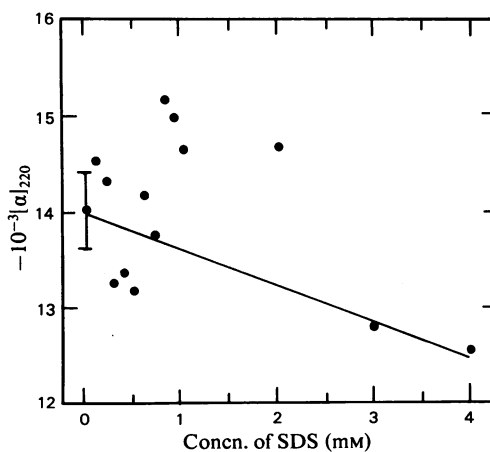


Fig. 2. *Effect of SDS on collagen fibril formation at 30 mM-Na₂HPO₄*

Fibril formation was conducted at 26°C in buffer containing 30 mM-Na₂HPO₄, 200 mM-NaCl, 30 mM-Tes, 0–1.0 mM-SDS, 2.5 mM-acetic acid and 0.1 mg of collagen/ml, final pH 7.0–7.2. Full fibril inhibition occurs at 0.5 mM-SDS and above. Fibril promotion occurs at 0.3 mM-SDS. Points in parentheses indicate that $t_{\frac{1}{2}}$ was greater than 900 min.



Fig. 4. *Effect of SDS on collagen tertiary structure*

Measurements were taken at 220 nm at room temperature with a Cary 60 o.r.d. spectropolarimeter. The sample contained 0.1 mg of collagen/ml, 30 mM-Tes, 30 mM-Na₂HPO₄ and 0.1–4.0 mM-SDS. At 0.3 mM-SDS the loss of collagen structure is calculated to be about 10%.

values decrease from $-14\,200\,\text{degree}\cdot\text{ml}\cdot\text{dm}^{-1}\cdot\text{g}^{-1}$ at 0 mM-SDS to $-13\,200\,\text{degree}\cdot\text{ml}\cdot\text{dm}^{-1}\cdot\text{g}^{-1}$ at 0.5 mM-SDS then increase to $-15\,200\,\text{degree}\cdot\text{ml}\cdot\text{dm}^{-1}\cdot\text{g}^{-1}$ at 0.8 mM-SDS and again decrease to $-13\,000\,\text{degree}\cdot\text{ml}\cdot\text{dm}^{-1}\cdot\text{g}^{-1}$ at 4.0 mM-SDS. This pattern was also seen at slightly higher collagen concentrations. Although the scatter in the data is large, the decrease in the specific rotation value at 0.3 mM-SDS represents a loss of helical structure of no more than a few per cent, probably less than 10%.

*Electron micrographs*

Fig. 5 illustrates the native-type fibrils formed in the control and sample buffers at a magnification of $\times 42\,000$. The fibrils formed in the control buffer (Fig. 5a) appear to be similar to or identical with fibrils *in vivo*. The characteristic negative staining pattern, consisting of a fine polarized band pattern superimposed on alternating regions of light and dark bands, is seen in the control fibrils, with a D period measured at 68 nm. At a magnification of $\times 12\,500$, the fibrils are long, with ends having the usual tactoid taper (Williams *et al.*, 1978). Fibrils have a constant diameter throughout their lengths, with bends a result of shear during grid preparation.

Fibrils formed in 0.04 mM-SDS (Fig. 5b) are banded similarly to the native form and have a measured D period of 65 nm. These fibrils, however, tend to be less well packed than controls, and diameters increase at areas of loose lateral packing. Evidence of 'unravelling' is also seen.

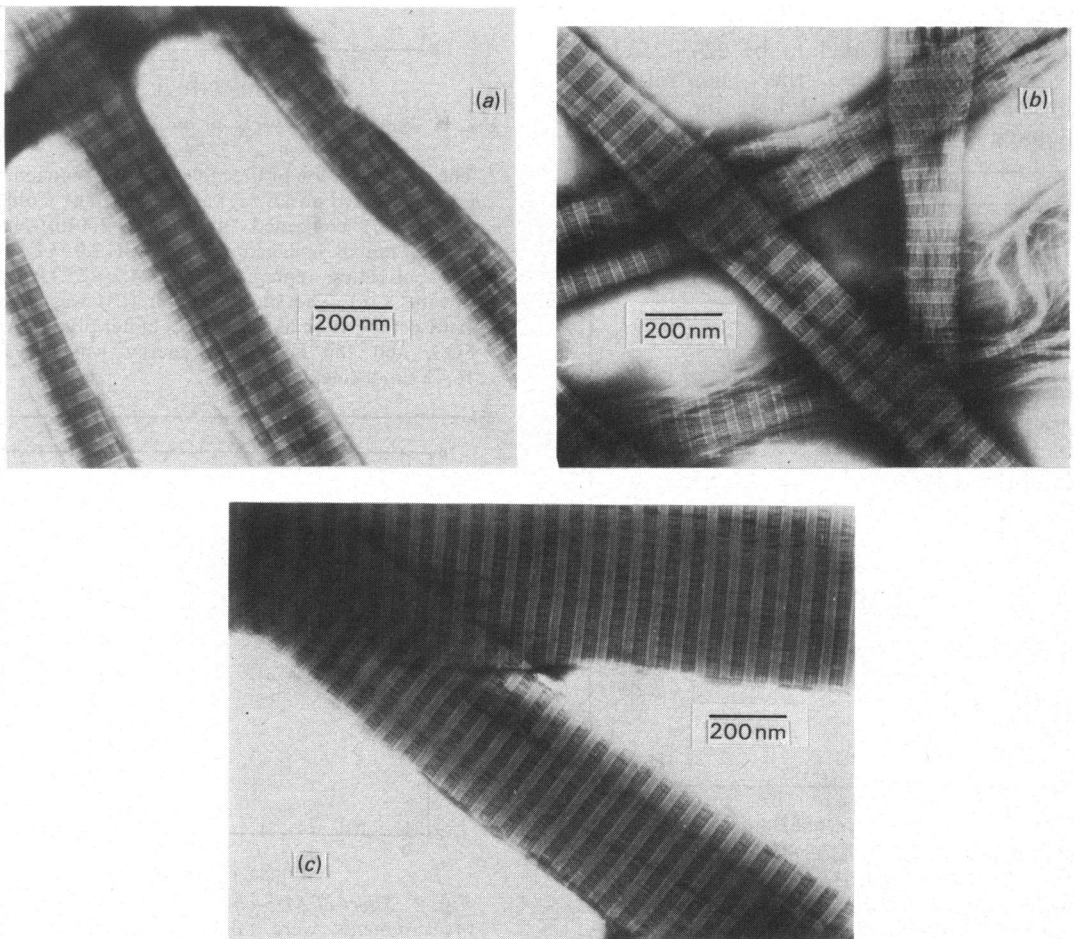Fibrils formed in 0.3 mM-SDS (Fig. 5c) are also



Fig. 5. *Electron micrographs of negatively stained collagen fibrils*

Preparation is discussed in text. Fibrils formed in control buffer (a) had the native banding pattern with a D period of 68 nm, those in 0.04 mM-SDS (b) contained a D period of 65 nm and those in 0.3 mM-SDS (c) contained a D period of 65 nm.
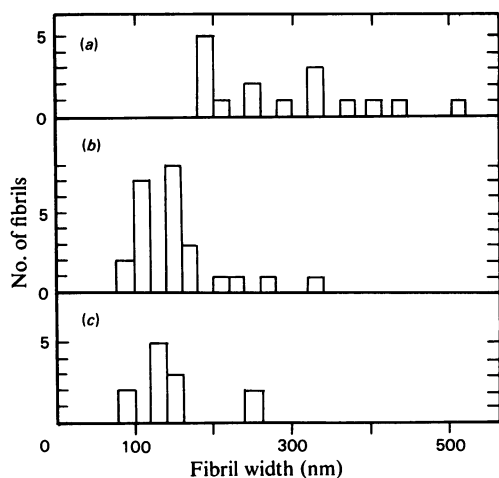
Fig. 6. *Population histograms of collagen fibril widths*
Fibrils formed in the presence of (*a*) 0.3 mM-SDS
measured $291 \pm 103$ nm and (*b*) 0.04 mM-SDS
measured $158 \pm 56$ nm, and controls (*c*) measured
$149 \pm 52$ m (means $\pm$ s.D.).

banded similarly to the native form and contain a
measured D period of 65 nm. These fibrils appear
to be well formed with no loosely packed or
'unravelled' portions. Fibril diameters are 2–3-fold
larger than those seen in the control or 0.04 mM-
SDS formation buffers.

Population histograms of fibril widths are
presented in Fig. 6. As a method of comparison,
the mean width $\pm 1$ s.D. is determined for each
sample. The control fibrils measured $149 \pm 52$ nm.
Those formed in the 0.04 mM-SDS buffer were
similar in size, $158 \pm 56$ nm, whereas those formed
in 0.3 mM-SDS measured $291 \pm 103$ nm.

## Discussion

The inhibition of fibril formation by excess
anion has previously been reported for phosphate
(Williams *et al.*, 1978): at 30 mM-phosphate, $t_{\frac{1}{2}}$
values were measured at about 80 min and rose
smoothly to 375 min at 50 mM-phosphate. In com-
parison, SDS is at least 100 times more potent,
since full fibril inhibition occurred at 0.5 mM-SDS.
The difference between the effects of the two
anions resides in their relative strength of associ-
ation to collagen and their ability to structure bulk
water. Collagen fibril formation is an endo-
thermic process involving the formation of hydro-
phobic and electrostatic interactions between
adjacent molecules, with the accompanying release
of associated water molecules (Cassel, 1966). The
fibril process is promoted by agents that increase
the disorder of the bulk water, such as increasing
solution temperature or addition of ions that

'break' water structure (Cooper, 1970). By con-
trast, fibril formation is retarded by agents that
increase the order of the bulk water, such as
lowering solution temperature or addition of ions
that 'make' water structure. Phosphate and sul-
phate ions are 'makers' of water structure (Cooper,
1970). The inhibitory effect of phosphate ion is
probably due to its effects both in structuring bulk
water and in disrupting electrostatic interactions
between collagen molecules. The relative impor-
tance of the two effects should differ, since the
0.2 M-NaCl concentration used in the present
studies will make the role of general electrostatic
interactions a small one. It is still possible that
specific electrostatic interactions could be disrupt-
ed, but overall it seems more probable that the
effects of structuring water plays the largest role in
phosphate-induced inhibition of fibril formation.
This effect is gradual and concentration-depen-
dent, since a phase change is not possible for the
anion.

Unlike phosphate, SDS-induced inhibition of
fibril formation is not gradual, but is sharp, is co-
operative and occurs at 100-fold lower concentra-
tion. The potency of SDS can be partially
explained by its ability to structure water with both
its polar head group and its hydrophobic tail. It is
known that the fibril formation is inhibited by
ionic and non-ionic surfactants (Hayashi & Nagai,
1973; Honya & Mizunuma, 1974; Suarez *et al.*,
1980). Those studies support the idea that increas-
ing the length of the hydrocarbon chain increases
potency of the inhibitor. Those studies also suggest
that the role of the inhibitor is predominantly one
of weakening hydrophobic interactions between
collagen molecules. Whether the inhibitory effect
of surfactants is due to a general increase in water
structure or to a general disruption of hydrophobic
interactions between collagen molecules, it seems
that the inhibition curve would be a smooth
transition. It is, however, not a smooth transition,
but a sharp one. This indicates that a small
increase in SDS concentration, from 0.4 mM to
0.5 mM, causes a large increase in the number of
collagen molecules unfit to self-assemble. The
general denaturation of collagen by SDS exhibits a
non-linear response to SDS concentration (Freytag
*et al.*, 1980); however, the o.r.d. studies in the
present work indicated that the collagen molecules
are 90–95% native at the SDS concentration where
fibril inhibition occurs.

It is suspected that the sharp transition of the
SDS inhibition curve represents a specific denatur-
ation of the telopeptides. It is also suspected that in
the α1(I) chain the *N*-terminal is more susceptible
to SDS than is the *C*-terminal telopeptide. This is
based on the greater number of stabilizing hydro-
phobic residues in the condensed structure of the

C-terminal (Capaldi & Chapman, 1982) than in the 'hairpin' structure of the N-terminal (Helseth *et al.*, 1979).

The idea of the N-terminal susceptibility to SDS is supported by the formation of large fibrils at 0.3 mM-SDS. Decreasing the number of native N-terminal telopeptides will decrease the number of fibril nucleation centres and also decrease the number of collagen molecules able to participate in linear associations. This will not, however, decrease the ability of individual collagen molecules to participate in fibril formation. These events are considered to be regulated by the proximal region of the C-terminal. The lateral events will continue and large fibrils will result. This model is based on the observation that large (400 nm wide) fibrils banded similarly to the native form are formed after 18 h digestion with pepsin (Capaldi & Chapman, 1982), which removes part or all of the N-terminal and the distal portions of the C-terminal telopeptides.

A difference between collagen treated with low concentrations of SDS and pepsin-treated collagen is the rate of fibril formation. The enzyme treatment results in increases in the lag phase. No increases are seen at the SDS concentration where large fibrils are formed, namely 0.3 mM. A speculative reason for this involves the presence of an intact distal portion of the C-terminal in the α1(I) chain. This region and the N-terminal telopeptide are lost or degraded in digestion with pepsin. In the presence of SDS, only the N-terminal is denatured. This allows the distal region of the C-terminal to direct nucleation and linear growth events at a faster rate than in pepsin-treated collagen. That the rate in SDS can be as fast as or faster than the fibril formation rates in control buffers may have to do with differences in the lag time.

The lag time of SDS-promoted fibril formation probably produces fewer nucleation centres than does the lag time of controls. This decrease is the result of the suspected SDS effect on the N-terminal telopeptide. With a decrease in the normal direction of lag-phase events, the lateral growth-phase events begin earlier. This is seen as a promotion of fibril formation, since turbidity only measures the onset and rate of lateral associative events.

The activation energies of the fibrillar process in the control buffer and in 0.3 mM-SDS-containing buffers were found to be approximately the same. This also supports the idea that the C-terminal is not affected by SDS at this concentration. Calculated from the temperature-dependent changes in the $t_{\frac{1}{2}}$ value of the turbidity growth phase, the activation energy measures the lateral associative events (Comper & Veis, 1977; Helseth & Veis, 1981).

It is probable that the effect of sulphate-containing matrix elements on fibrillogenesis *in vivo* is to offer a degree of regulation by direct association to the collagen molecule. The site of this control may be the N-terminal telopeptide of the α1(I) chain.

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