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The promotion of collagen polymerization by lanthanide and calcium ions

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Ca^{2+} (1–5 mM) and lanthanide (20–250 μM) ions enhance the rate of polymerization of purified calf skin collagen (1.5 mg/ml) at pH 7.0 in the presence of 30 mM-Tris/HCl and 0.2 M-NaCl. Both the nucleation phase and the growth phase of polymerization are accelerated. The activation energy of the growth phase, 239.3 ± 24.3 kJ/mol (57.2 ± 5.8 kcal/mol), is decreased to 145.6 ± 9.6 kJ/mol (34.8 ± 2.3 kcal/mol) by 5 mM-Ca^{2+} and to 75.3 ± 4.6 kJ/mol (18.0 ± 1.1 kcal/mol) by 25 μM-Sm^{3+}. In contrast, the activation energy of the nucleation phase, 191.6 ± 23.4 kJ/mol (45.8 ± 5.6 kcal/mol), is only slightly decreased by Ca^{2+} or Sm^{3+}. Collagen fibrils formed in the presence of Sm^{3+} are thinner than control fibrils, and more thermoresistant.

Collagen polymerization, or 'gelling', is a spontaneous extracellular process essential to the integrity of connective tissues. It follows the cleavage of non-helical extension peptides from procollagen molecules secreted by connective-tissue cells. The resulting polymeric collagen fibrils are more thermostable, more resistant to enzymic attack and less soluble than are the monomers. Spontaneous inter- and intra-molecular cross-linking further stabilizes and insolubilizes the fibrils, both *in vitro* and *in vivo* during maturation and aging. Despite the physiological importance of these processes, much of their biochemistry remains unknown.

Solutions of collagen molecules become turbid as they polymerize *in vitro*, a circumstance that forms the basis of a convenient method of studying collagen gelling. Such studies reveal gelling to be a biphasic process (Wood & Keech, 1960; Wood, 1960; Cassel *et al.*, 1962; Comper & Veis, 1977*b*; Williams *et al.*, 1978). An initial nucleation phase, during which the turbidity of the collagen solution remains low, precedes a growth phase, where the turbidity quickly increases. The nucleation phase seems to represent the formation of linear aggregates of collagen molecules, whereas the growth phase reflects stepwise lateral growth (Silver & Trelstad, 1979).

Although polymerization progresses spontaneously, it is sensitive to changes in pH, ionic strength, temperature and collagen concentration. Gelling is accelerated by lowering the pH from 8.0 to 6.0, or the ionic strength from 0.31 M to 0.13 M (Wood & Keech, 1960). The rate of gelling also increases as the concentration of collagen increases (Wood & Keech, 1960). The reaction is highly temperature-sensitive. Polymerization is extremely slow below 20°C, and at temperatures above about 39°C (depending on the other ambient conditions) collagen molecules denature.

In the present investigation, we have examined the effects of Ca^{2+} on the gelling of calf skin collagen, at 37°C and ionic strengths approximating to those of extracellular fluids *in vitro*. Ions of the lanthanide series (Ln^{3+}), which often serve as isomorphous replacements for Ca^{2+} in biochemical systems (Williams, 1979), have also been used. That Ln^{3+} might prove useful in the present context is suggested by previous work from this laboratory (Evans & Mears, 1980; Evans & Tew, 1981; Evans, 1981).

Experimental

Materials

Pepsin-solubilized calf skin collagen was obtained from the Collagen Corp. (Palo Alto, CA, U.S.A.) as a sterile solution (approx. 3 mg/ml) in 10 mM-HCl, pH 2. This product forms firm gels (see below), which are trypsin-resistant but collagenase-sensitive (results not shown). The various lanthanide chloride hexahydrates were purchased from Alfa Products (Danvers, MA, U.S.A.). Carbon-coated 200-mesh copper grids for electron microscopy were obtained from E. F. Fullam (Schenectady, NY, U.S.A.). Other chemicals and supplies were purchased from standard sources.

Lanthanide solutions

Stock solutions (10 mM) were made in distilled water, and the lanthanide concentrations were confirmed by titration with a standard solution of EDTA with murexide as indicator.

Collagen polymerization studies

Solutions of collagen in HCl were dialysed overnight against a large volumetric excess of 30mM-Tris/HCl buffer, pH 7.0, containing 0.2M-NaCl. As storage of the collagen after dialysis led to a rapid decline in the rate and extent of gelling, polymerization studies were always made with freshly dialysed samples. Turbidometric analyses were made with a Beckman model 34 recording spectrophotometer having a thermostatically controlled cuvette holder. Freshly dialysed collagen (0.5 ml), 30mM-Tris/HCl buffer, pH 7.0, containing 0.2M-NaCl (0.5 ml) and 25 μl of the appropriate concentration of Ca^{2+} or Ln^{3+} (aqueous) or distilled water were mixed in a disposable plastic semi-micro cuvette with a light-path of 1 cm, and the A_{500} of the solution was constantly monitored. The gelling curves thus obtained were analysed by reference to three parameters: the length of the nucleation phase (t_n), the maximum rate of increase of turbidity during the growth phase (dA/dt) and the A_{500} obtained after completion of gelling ($A_{max.}$) (Fig. 1).

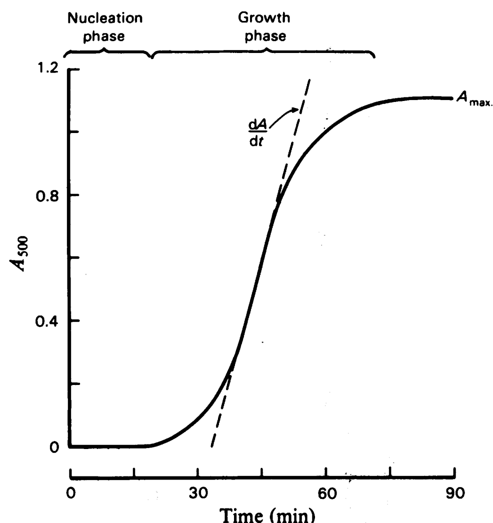

Fig. 1. *Typical gelling curve for calf skin collagen (1.5 mg/ml) at 37°C in 30mM-Tris/HCl buffer, pH 7.0, containing 0.2M-NaCl*
For experimental details see the text.

Collagen fibril thermostability

To assess the thermostability of the collagen polymers formed under the various conditions, the temperature of the thermostatically controlled cuvette holder was raised to 45°C, after the attainment of the $A_{max.}$ at 37°C. This invariably caused a slight elevation in the $A_{max.}$, followed by a protracted decline as the gel denatured. The rate of denaturation was measured as the initial rate of decline in A_{500}.

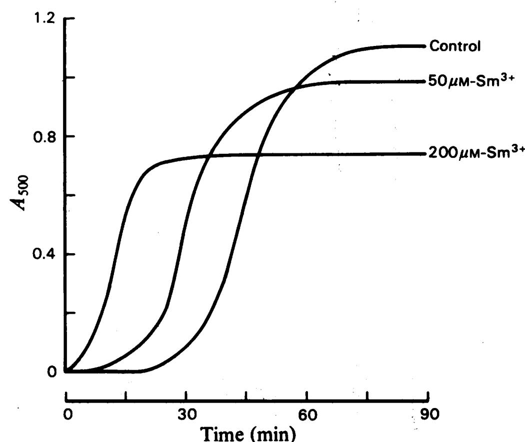

Fig. 2. *Acceleration of collagen polymerization by 50 μM- and 200 μM-Sm^{3+} at 37°C*
For experimental details see the text.

Electron microscopy

A sample of the collagen gel was placed on a carbon-coated copper grid and immersed in aq. 1% (w/v) phosphotungstic acid for 10 min. The grids were then removed, air-dried and examined with a Phillips EM 300 transmission electron microscope.

Results

Polymerization properties of calf skin collagen

A typical sigmoidal gelling curve is shown in Fig. 1. The nucleation phase of 16 min ($t_n = 16$) precedes a phase of fibril growth, during which time the solution of collagen becomes increasingly turbid, producing a maximum rate of increase in A_{500} of 0.05 unit/min ($dA/dt = 0.05$). At the conclusion of gelling, the solution has an A_{500} of 1.1 units ($A_{max.} = 1.1$). This pattern was reproducible, although the t_n, dA/dt and $A_{max.}$ all varied among different batches of collagen. Storage of the collagen also affected these parameters, generally leading to longer nucleation phases, lower rates of turbidity increase and lower maximum absorbances. Gelling curves were far more reproducible when conducted at the same sitting. For this reason, we were careful to perform comparative experiments at one session using a given batch of collagen.

EDTA and EGTA (1 mM) had no effect on the gelling curves (results not shown).

Effects of Sm^{3+} on collagen polymerization

Of the different Ln^{3+} ions employed, Sm^{3+} is the closest in ionic radius to Ca^{2+} and was the first tested for an effect on collagen polymerization. As shown in Fig. 2, 50 μM- and 200 μM-Sm^{3+} pro-

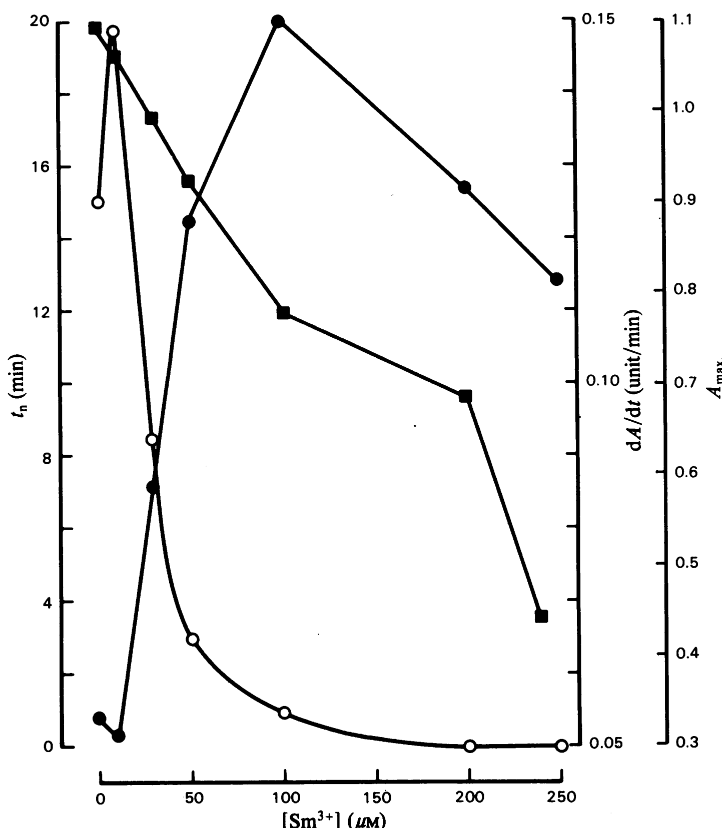

Fig. 3. *Effect of Sm^{3+} on the t_n (○), dA/dT (●) and $A_{max.}$ (■) of collagen polymerization*
For experimental details see the text.

gressively shortened the t_n, raised the dA/dt and lowered the $A_{max.}$. Examination of the effects of Sm^{3+} over a range of concentrations from 10 μM to 250 μM revealed a strong concentration-dependence (Fig. 3). Interestingly, the lowest concentration (10 μM) of Sm^{3+} slightly lengthened the t_n and lowered the dA/dt. However, at all higher concentrations, there was a marked acceleration of both nucleation and growth. The nucleation phase was eliminated at concentrations of Sm^{3+} above 100 μM; this was also the concentration of Sm^{3+} giving the maximum acceleration of the growth phase.

Electron-microscopic examination of the fibrils revealed that increasing concentrations of Sm^{3+} resulted in the formation of progressively thinner fibrils (Table 1).

Table 1. *$A_{max.}$ and thickness of collagen fibrils formed in the absence and in the presence of Sm^{3+}*
Gels were formed at 37°C at pH 7.0. Collagen concentration was 1.5 mg/ml in the presence of 0.2 M-NaCl, as described in the text. Fibril thickness was measured by electron-microscopic examination. For experimental details see the text.

Concn. of Sm^{3+} (μM)	$A_{max.}$	Average fibril thickness (nm) (mean ± S.E.M.)
0	1.10	320 ± 62
25	0.98	210 ± 49
50	0.92	130 ± 31
100	0.77	78 ± 17

Effects of other Ln^{3+} ions on collagen polymerization

Lu^{3+}, Er^{3+} and La^{3+} were all tested in the manner of Sm^{3+}. All slightly extended the nucleation phase at low concentrations (10 μM) but greatly shortened it at higher concentrations. Examination of Fig. 4(*a*) reveals Sm^{3+} to be the most effective of the Ln^{3+} ions tested, and La^{3+} the least effective. Those lanthanides that produced the least shortening in the t_n at high concentration produced its least extension at low concentration, and vice versa.

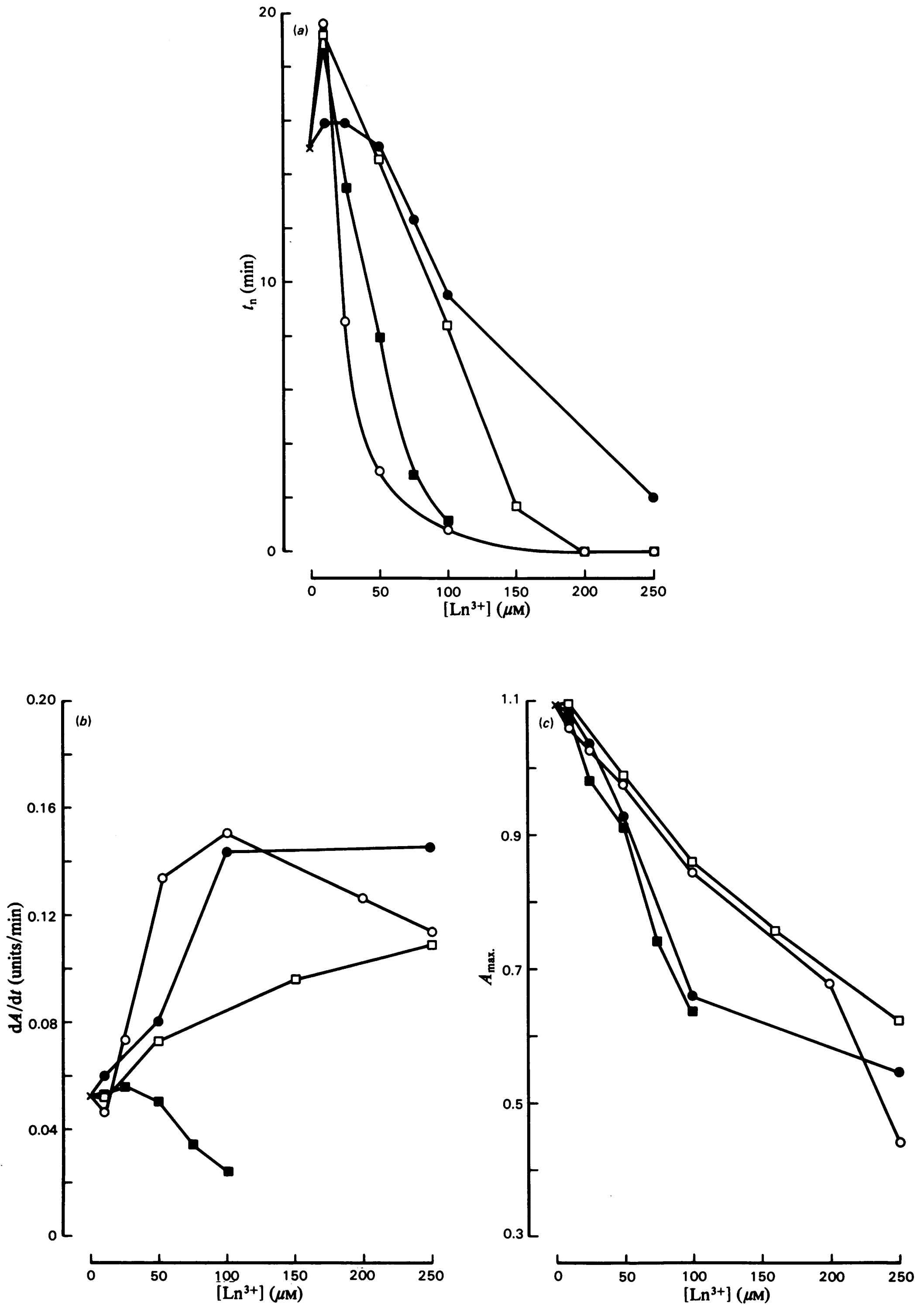

Fig. 4. *Effects of* Lu^{3+} (■), Er^{3+} (□), Sm^{3+} (○) *and* La^{3+} (●) *on (a)* t_n, *(b)* dA/dt *and (c)* $A_{max.}$ *of collagen polymerization*

For experimental details see the text.

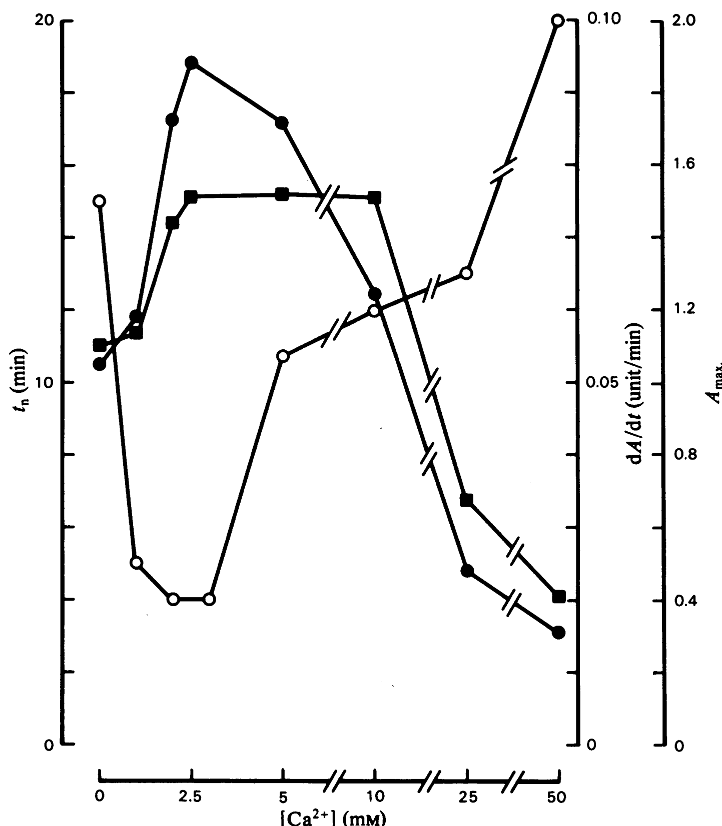

Fig. 5. *Effect of Ca^{2+} on t_n (○), dA/dt (●) and $A_{max.}$ (■) of collagen polymerization*
For experimental details see the text.

Er^{3+} and La^{3+} both accelerated the growth phase, La^{3+} being as effective as Sm^{3+}, but Er^{3+} less so. Lu^{3+}, in contrast, inhibited growth of the collagen fibrils, at concentrations above 50 μM (Fig. 4*b*).

All four Ln^{3+} ions lowered the $A_{max.}$ of the collagen fibrils in a qualitatively similar manner (Fig. 4*c*). Lu^{3+} was the most effective, and Er^{3+} the least.

Effects of Ca^{2+} on collagen polymerization

Ca^{2+} also shortened the nucleation phase and enhanced the rate of fibril growth (Fig. 5). The concentrations of Ca^{2+} needed to enhance collagen polymerization were two orders of magnitude greater than those of the Ln^{3+} ions producing a similar effect. Maximum shortening of the lag phase and enhancement of dA/dt were observed at Ca^{2+} concentrations around 2.5 mM, which is close to the physiological concentration of calcium in the serum. Concentrations of Ca^{2+} above 10 mM markedly inhibited polymerization. Unlike Ln^{2+} ions, Ca^{2+}, at concentrations that accelerated the rate of polymerization, elevated the $A_{max.}$.

Activation energies for collagen fibril nucleation and growth in the presence and in the absence of Ca^{2+} or Sm^{3+}

Arrhenius plots were constructed to determine the activation energies (E_a) of the nucleation and growth phases of collagen polymerization in the presence and in the absence of Ca^{2+} or Sm^{3+} (Figs. 6*a* and 6*b*).

The E_a for the nucleation phase was 191.6 ± 23.4 kJ/mol (45.8 ± 5.6 kcal/mol) (mean ± S.E.M., $n = 4$). Neither 5 mM-Ca^{2+} [$E_a = 159.8 \pm 18.0$ kJ/mol (38.2 ± 4.3 kcal/mol) ($n = 3$)] nor 25 μM-Sm^{3+} [$E_a = 159.0 \pm 9.6$ kJ/mol (38.0 ± 2.3 kcal/mol) ($n = 3$)] greatly affected this value (Fig. 6*a*). In contrast, the E_a of the growth phase, 239 ± 24.3 kJ/mol (57.2 ± 5.8 kcal/mol) ($n = 4$) was decreased to 145.6 ± 9.6 kJ/mol (34.8 ± 2.3 kcal/mol ($n = 3$) by 5 mM-Ca^{2+} and to 75.3 ± 4.6 kJ/mol (18.0 ± 1.1 kcal/mol) ($n = 3$) by 25 μM-Sm^{3+} (Fig. 6*b*).

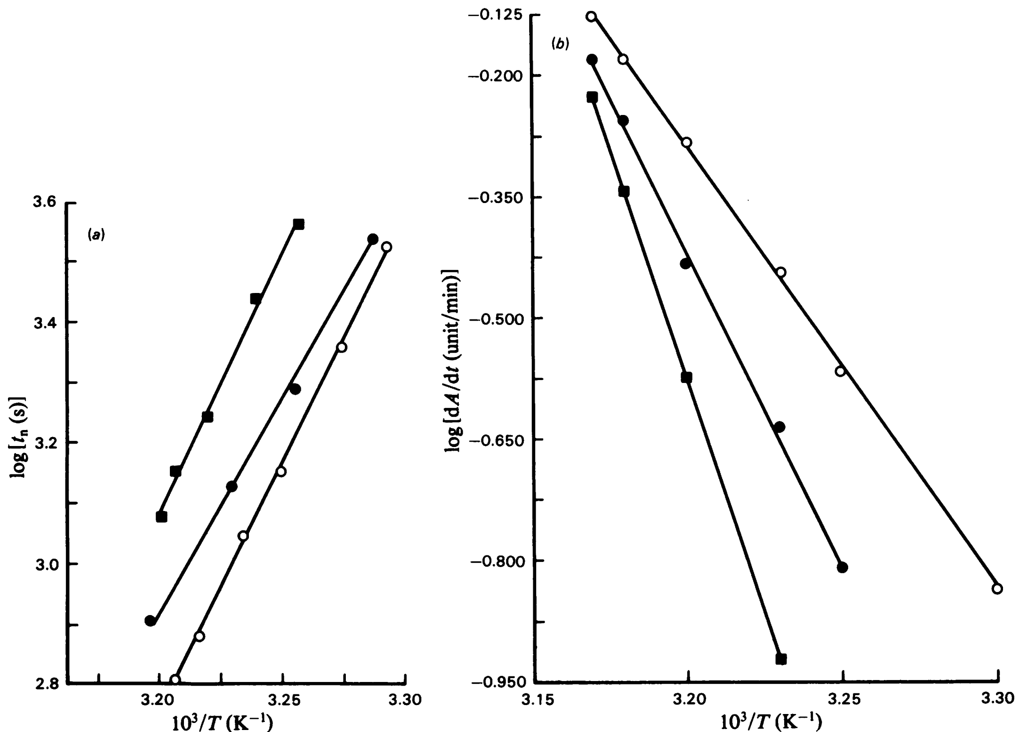

Fig. 6. *Arrhenius plots of (a) the nucleation phase and (b) the growth phase of collagen polymerization under control conditions (■), with 5 mM-Ca^{2+} added (●) and with 25 μM-Sm^{3+} added (○)*
For experimental details see the text.

Thermostability of collagen fibrils formed in the presence and in the absence of Sm^{3+}

During the experiments to determine the E_a for collagen polymerization, it was noted that, whereas collagen would normally fail to gel at temperatures above 39°C, gelling in the presence of Sm^{3+} could occur at temperatures as high as 42°C. These observations suggest that collagen is thermostabilized by Sm^{3+}. To test this possibility, gels were formed at 37°C in the presence and in the absence of Sm^{3+} and the thermostat controlling the cuvette holder was then switched to 45°C. This produced a decline in $A_{max.}$ as the fibrils depolymerized. The rate of depolymerization was lower in the presence of Sm^{3+} (Fig. 7).

Discussion

The interaction of Ca^{2+} with collagen is of considerable biological interest. Not only is it crucial to the formation of the hard connective tissues, bone and teeth, but calcification of the soft connective tissues occurs during aging. The present data suggest a regulatory role for Ca^{2+} at an early stage in the formation of such tissues, namely in the polymerization of newly synthesized collagen molecules.

The ability of physiological concentrations of Ca^{2+} to promote the polymerization of collagen under conditions of ionic strength, pH and temperature found *in vivo* suggests such a role for Ca^{2+} *in situ*. That low concentrations of Ln^{3+} ions mimic the effects of Ca^{2+} in an exaggerated manner is consistent with many other reports of this kind (e.g. Takata *et al.*, 1966; Darnall & Birnbaum, 1973; Evans, 1981).

Lowering of the E_a explains the acceleration of the growth phase of collagen polymerization by Ca^{2+} and Sm^{3+}. However, these cations have little effect on the E_a of the nucleation phase, which precedes fibril growth. This dichotomy reinforces the received view that nucleation and growth are two quite different processes, a position further supported by the ability of Lu^{3+} to accelerate nucleation although inhibiting fibril growth.

Our value of 239.3 ± 24.3 kJ/mol (57.2 ± 5.8 kcal/mol) for the E_a of fibril growth compares with the values of 243 kJ/mol (58 kcal/mol) (Williams *et al.*, 1978) for rat tail tendon and 113 kJ/mol (27 kcal/mol) (Wood, 1960) and 96–146 kJ/mol (23–35 kcal/mol) (Bensusan & Hoyt, 1958) for calf skin collagen. The E_a of the polymerization of native acid-soluble collagen molecules is not greatly affec-

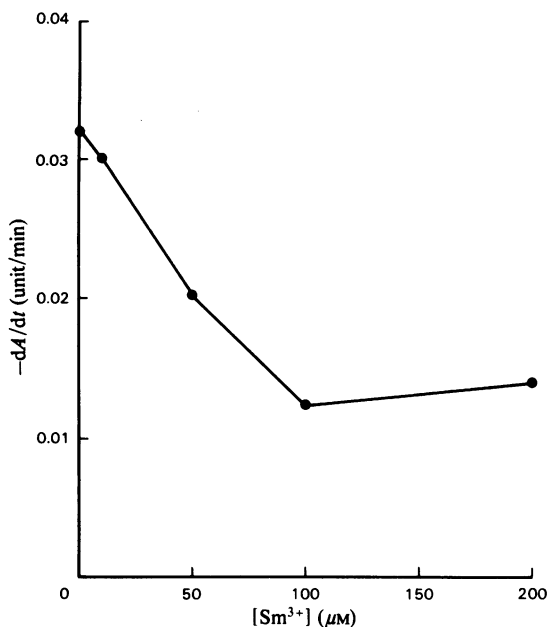

Fig. 7. *Effect of Sm^{3+} on the rate of thermodenaturation of calf skin collagen fibrils*

Fibrils were formed at 37°C in the presence of the concentrations of Sm^{3+} indicated. The fibrils were then heated at 45°C and the rate of decline in A_{500} was measured. For experimental details see the text.

ted by pH or ionic strength (Bensusan & Hoyt, 1958). It is likely that the high E_a value for our pepsin-solubilized preparation of calf skin collagen results from its attenuated telopeptide region. Comper & Veis (1977*a*) determined an E_a of 161.9kJ/mol (38.7kcal/mol) for acid-soluble rat skin collagen, but an E_a of 195.0kJ/mol (46.6kcal/mol) for its pepsin-treated derivative. It may be noted that, in addition to producing polymerization at temperatures above and below those at which collagen solutions otherwise failed to gel, Ln^{3+} ions induced gelling in 'aged' solutions of collagen that had lost their ability to polymerize spontaneously. This suggests that the transition from the intact triple helix to a denatured random coil is a progressive process with subtle intermediate forms, rather than a single all-or-none, step.

The marked dependence of collagen polymerization rates on pH and ionic strength have led several authors to conclude that ionic interactions are an important part of the process (Wood & Keech, 1960; Cassel *et al.*, 1962; Williams *et al.*, 1978). Although the identity of the charged groups involved has not been established, the preference of Ca^{2+} and Ln^{3+} ions for oxygen-donor ligand atoms is suggestive of the involvement of carboxy groups of acidic amino acid residues in the collagen chains. Experiments involving chemically modified collagen have revealed that carboxy groups are the main binding sites of Al^{3+} (Hörmann, 1974). The 'bridging' properties of Ca^{2+} and Ln^{3+} ions (Williams, 1979) could foster interaction between different collagen molecules during polymerization, and stabilize the resulting fibrils. Studies by Wood (1962) show that the more-cross-linked species of collagen are precipitated more rapidly and more completely than are the less-cross-linked species. Such a role is suggested by the work of Steven (1967), who showed that bovine extensor tendons become dispersable in 0.2M-acetic acid if pretreated with EDTA at pH 7.5. Re-addition of Ca^{2+} eliminated this effect. The enhanced thermostability of collagen fibrils formed in the presence of Sm^{3+} may have a similar basis. It may be noted that Adam *et al.* (1964) have postulated a similar collagen-stabilizing role for anti-arthritic drugs containing gold.

Cooper (1970) has suggested that the effects of small solute molecules on the polymerization of collagen can be explained by their structural effects on liquid water. Ions can be classified as 'makers' or 'breakers' of water structure (Kavanau, 1964). Cooper's (1970) suggestion that structure-makers inhibit collagen fibril formation is contradicted by our studies: both La^{3+} and Ca^{2+} are structure-makers, with La^{3+}, the more stimulatory of the two, being a more efficient structure-maker (Kavanau, 1964). At first sight, our data also appear to contradict those of Bensusan & Hoyt (1958), who reported that Ca^{2+} inhibited collagen fibril formation. However, their studies used a low-ionic-strength buffer, and their observations may have reflected the increase in the total ionic strength on adding Ca^{2+}; as noted above, the rate of polymerization is inversely related to ionic strength. Similarly, reports such as those by Von Hippel & Wong (1963) and Weinstock *et al.* (1967), of a destabilizing effect of Ca^{2+}, use a supraphysiological concentrations (0.12–1M) of Ca^{+}.

Collagen fibril width is negatively correlated with the concentration of Sm^{3+} and the $A_{max.}$ (Table 1). It was previously found that fibrils formed under conditions of ionic strength (Wood & Keech, 1960) or in the presence of certain polyanions (Wood, 1960) that accelerate gelling were thinner and of lower $A_{max.}$ than control fibrils. This suggests that Sm^{3+} itself does not lead to the formation of thinner fibrils, but rather that they are a consequence of the shorter gelling times. Both sets of data support the position that the increase in turbidity reflects the lateral growth of fibrils and that the $A_{max.}$ is determined by their final width.

The spectroscopic properties of various Ln^{3+} ions (Reuben, 1975) facilitate biochemical investigation

of Ca^{2+}-binding sites on many proteins. The superiority of Sm^{3+}, the Ln^{3+} ion of closest radius to Ca^{2+}, over La^{3+}, Er^{3+} and Lu^{3+} in shortening of the nucleation phase (Fig. 4*a*) raises the possibility of these being a sterically restricted Ca^{2+}-binding site important in nucleation (Tew, 1977; Evans, 1981). No such steric restraint appears to operate on the binding site(s) that affect(s) the rate of fibril growth (Fig. 4*b*).

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