# THE INTERACTION OF SILICIC ACID WITH COLLAGEN AND GELATIN MONOLAYERS

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Silicic acid reacts with monolayers of collagen and gelatin between pH 3 and 9. The films are tanned in this pH range and the force/area curves after a second compression are not identical with those relating to the first compression. Tanning is probably due to the cross-linking of chains or parts of protein chains rather than to the polymerization of silicic acid beneath the film, as is the case with globular proteins.

The previous paper described studies on the interaction of silicic acid with films of several proteins, which are normally globular. This communication discusses the results obtained in a parallel study of the interaction of silicic acid with films of collagen and its degradation product, gelatin. The fibrous proteins are not easy to study because it is difficult to bring them into solution without alteration. Native collagen is insoluble in most solvents. A fraction of the collagen of tendon is soluble in acetic acid but very dilute solutions only can be prepared. Gelatin is degraded and is water-soluble. Hide powder collagen has been spread from anhydrous formic acid but it is stated to resemble gelatin rather than collagen. The properties of films of these three proteins have been examined.

## EXPERIMENTAL

### MATERIALS

The apparatus and the method of preparation of substrates are described in the previous paper. Results were plotted as force/area curves from which the surface pressures at an arbitrary area (0·5 $m^2$/mg) were read off. These values were plotted against the substrate pH to give force/pH curves. Force/pH curves are preferable to area/pH curves when the films are highly compressible.

Gelatin and hide powder collagen.—Gelatin (isoelectric point 5·0) and hide powder collagen (14·7 % N) were kindly provided by the British Leather Manufacturers' Research Association. The gelatin was spread from a solution (0·05 %) in 0·1 % aqueous ethanol and the hide powder collagen from anhydrous formic acid, on to spreading areas of 8·0 $m^2$/mg (temp. 25°).

Tendon collagen.—Tendon collagen was prepared from rat tails. The tendon was stripped from the tail, washed in normal saline, then treated with 0·1 % trypsin in normal saline for 24 h at 37°. The tendon was washed with water then with 10 % NaCl to remove proteins other than collagen. Fat and other surface-active substances were removed by ether and ethanol, the tendon finally being washed in acetone and dehydrated *in vacuo*. The dry tendon was then placed in 0·1 % acetic acid and allowed to dissolve for 12 h or more before the residue was filtered off. The filtrate containing 0·018 mg/ml gave satisfactory monolayers.

## RESULTS

### GELATIN AND HIDE POWDER COLLAGEN

The films of gelatin and hide powder collagen behaved in an almost identical manner.

### CONTROL SUBSTRATES (1 M NaCl + 0·0125 M $Na_2CO_3$)

All the films of gelatin and hide powder collagen were reversibly compressible (see, e.g., fig. 1, curve A). There was little variation with pH of the force required to maintain the film at a constant area (fig. 2 and 3). No maximum was observed at the isoelectric

point, pH 5·0. The collagen and gelatin spread rapidly equilibrium being reached in about 5 min.

UNPOLYMERIZED SILICIC ACID SUBSTRATES (1 M NaCl + 0·0125 M $Na_2CO_3$ + 0·002 M unpolymerized silicic acid)

Both the force/area curves (e.g. fig. 1, curve A) and the force/pH curve (fig. 2) were unchanged.

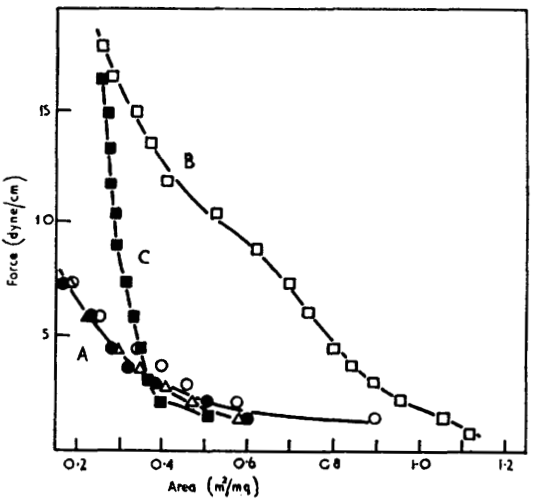


FIG. 1.—Force/area curves of hide powder collagen films, approximately pH 5.

A ○ control substrate, first compression (pH 4·9); ● control substrate, second compression (pH 4·9); Δ substrate containing unpolymerized silicic acid (0·002 M), first compression (pH 5·0).

B on substrate containing polymerized silicic acid, 0·002 M, first compression (pH 5·3).

C on substrate containing polymerized silicic acid, 0·002 M, second compression (pH 5·3).

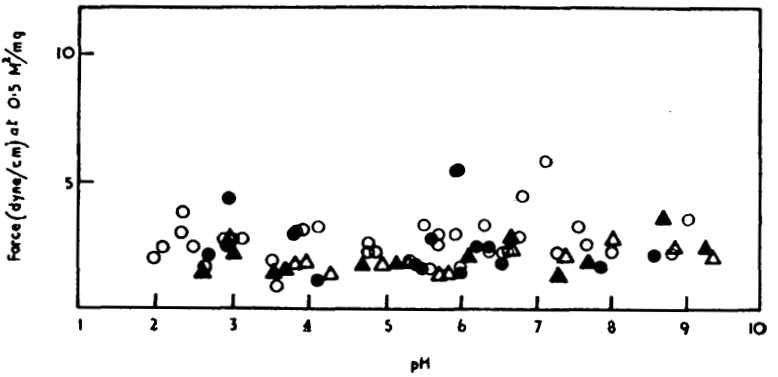


FIG. 2.—Force/pH plot of hide powder collagen films on control substrates compressed after ○ 0·5 min; ● 15 min, and on substrates containing unpolymerized silicic acid (0·002 M); compressed after Δ 5 min, ▲ 15 min.

POLYMERIZED SILICIC ACID SUBSTRATES (1 M NaCl + 0·0125 M $Na_2CO_3$ + prepolymerized silicic acid)

Fig. 1, curve B, is the force/area curve of a film spread on polysilicic acid at pH 5. The film was allowed to spread for 5 min before it was compressed. The protein had larger specific areas at all pressures in the presence of polysilicic acid. Curve C, relating to the second compression, is no longer coincident with curve B, showing that the specific areas were reduced.

The force/pH curves too are different when the film was on 0·002 M polysilicic acid (fig. 3). The force/pH curve is raised between pH 3 and 8 with a maximum in the region of pH 6·0. On more dilute silicic acid substrates there was a reduction in the specific area until at 0·0001 M, no increase in area was detected on the force/pH curve. However, the force/area curves relating to the first and the second compressions are not coincident, showing that interaction had still taken place even at this low concentration of silicic acid.

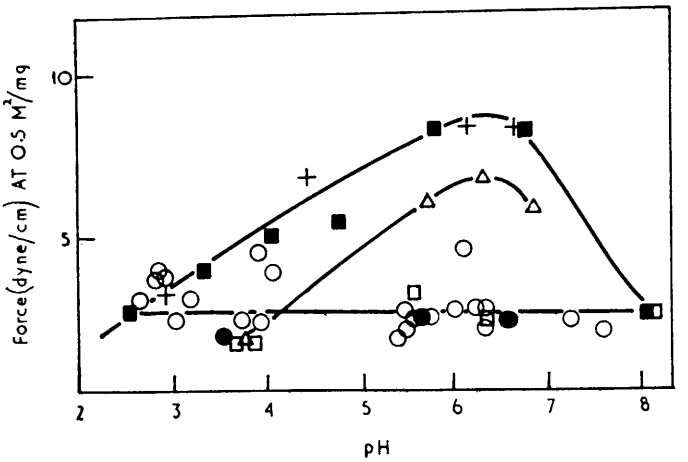


FIG. 3.—Force/pH curves of gelatin films on ○ control substrates and on substrates containing polymerized silicic acid: ■ 0·002 M; + 0·001 M; Δ 0·00025 M; □ 0·0001 M; ● 0·00005 M; compressed after 5 min.

## TENDON COLLAGEN

### CONTROL SUBSTRATES (1 M NaCl + 0·0125 M $Na_2CO_3$)

The force/area curves obtained for films spread on control substrates are similar to those of hide powder collagen and gelatin films. The curves relating to the first and second

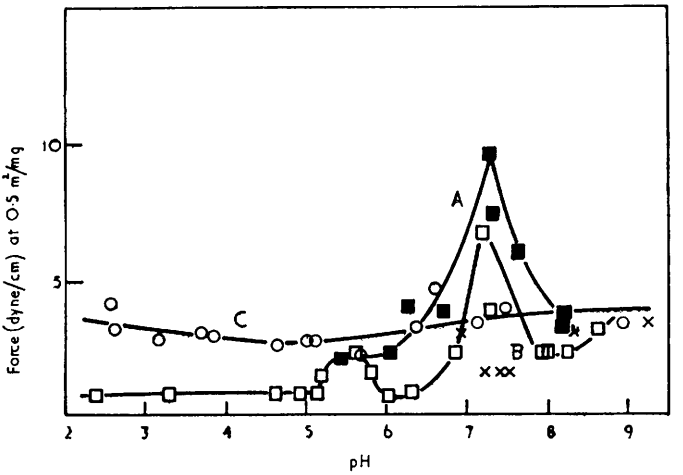


FIG. 4.—Force/pH curves of tendon collagen films on ○ control substrates compressed after 5 min and on substrates containing 0·002 M polymerized silicic acid compressed after □ 5 min and ■ 15 min. × on substrates containing 0·0005 M polymerized silicic acid compressed after 5 min.

compressions are again coincident and the force/pH curve (fig. 4, curve C), too, show the same form.

UNPOLYMERIZED SILICIC ACID SUBSTRATES (1 M NaCl + 0·0125 M $Na_2CO_3$ + 0·002 M unpolymerized silicic acid)

Again, unpolymerized silicic acid has no detectable effect on either the force/area curves or the force/pH curves.

POLYMERIZED SILICIC ACID SUBSTRATES (1 M NaCl + 0·0125 M $Na_2CO_3$ + 0·002 M polymerized silicic acid)

Like the force/area curves of gelatin and hide powder collagen those of tendon collagen are changed, showing increased specific areas on the initial compression but reduced areas on the second compression. The force/pH curve (fig. 4, curve B), however, is different from those of gelatin and hide powder collagen, no longer showing a maximum at pH 5·5

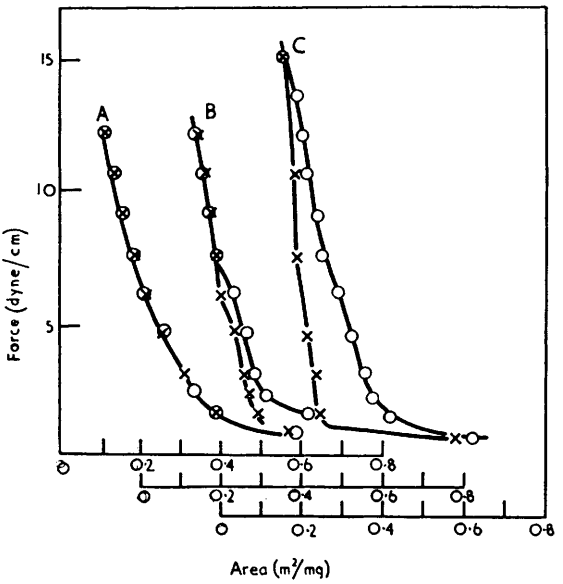


FIG. 5.—Force/area curves of tendon collagen on substrates containing polymerized silicic acid. A, pH 2·4; B, pH 3·3 and C, pH 4·6. ○ first compression, × second compression.

to 6·0, but at pH 7·0 to 7·5, near the iso-electric point of native collagen. In addition to this maximum there is a small peak in the region of pH 5·5. Except at pH values near to the maximum, there appeared to be inhibition of spreading, as shown by a reduction of the specific areas, at all pH values.

Apparently there is some type of interaction even if the pH is as low as 2·5. At pH 2·4 the force/area curves for a first and second compression are coincident, although somewhat steeper than those of a control, but at pH 3·3 they are no longer coincident (fig. 5). When the spreading time was increased from 5 to 15 min, the force/pH curve showed a merging of the two peaks (fig. 4, curve A), due to increased specific areas in the region of pH 6·0.

A decrease in the concentration or degree of polymerization of the silicic acid greatly reduced the effect on the specific area (fig. 4); this contrasts with the results obtained with gelatin and hide powder collagen where an effect is still observed at very low concentrations.

## DISCUSSION

The force/area curves which relate to gelatin and collagen films differ markedly from those of albumin, methaemoglobin and insulin films. These globular proteins give the usual curves, the steep parts of which cut the area axis at a point between 0·7 and 1·0 $m^2$/mg on extrapolation. The minimum interfacial area into which a protein can pack when only the backbone remains in the interface is calculated to be 0·7 $m^2$/mg.

Collagen and gelatin films are much more compressible than are those of globular proteins. Ellis and Pankhurst studied films of hide powder collagen at surface pressures below 1 dyne/cm and found true monolayers between 1·3 and 1·8 $m^2$/mg. The larger value agreed with the area they calculated the protein would occupy if the backbone and the side-chains were to lie horizontally in the interface. A change in the surface moment, observed when the film was compressed further, indicates that the molecules become re-orientated. The re-orientation is complete at an area of 1·3 $m^2$/mg, a value nearly twice that calculated for a protein film in which only the backbone remains in the interface.

This anomaly may be due to the proline residues of collagen. Peptides such as glycyl-prolyl-hydroxyproline have been isolated from collagen hydrolyzates, showing that proline groups may be adjacent in the protein chain. Such a sequence changes the direction of the protein backbone by 90°, introducing an irregularity which will prevent the close-packing possible in films of globular proteins.

At higher pressures collagen films are less compressible but they are still much more compressible than are films of globular proteins; areas as low as 0·5 $m^2$/mg are obtained by applying even small pressures, e.g. $< 5$ dyne/cm. Moreover, at high pressures the films show no collapse point even when the area is reduced to 0·3 $m^2$/mg. Bear suggested that collagen contains sequences of polar and non-polar regions, and this view is supported by the investigations of the amino-acid sequence. On compression, the non-polar sections of the chain would be expected to leave the aqueous phase until a folded film of considerable thickness is formed.

In contrast to films of globular proteins, the force required to bring a collagen or gelatin film to an arbitrary specific area showed very little variation with pH (fig. 2). Two factors may be involved. First, collagen has few ionizable groups so there is little repulsion between molecules at any pH; in contrast the area/pH curves of globular proteins rise at extreme pH values. Secondly, collagen is insoluble at all pH values so there are no minima in the force/pH curve at either side of the isoelectric point due to protein dissolving in the substrate. When collagen or gelatin films are compressed, allowed to expand, then re-compressed, the force/area curves relating to the two compressions are coincident which also indicates that the protein is not forced into the substrate.

When the solution beneath collagen or gelatin films contains silicic acid which has not been prepolymerized, the characteristics of the films are scarcely altered by the silicic acid. The films are still readily compressed, the force/pH curve is unchanged and the force/area curve relating to a second compression is still almost coincident with that relating to the first compression. Ellis and Pankhurst likewise found that polyhydric phenols of low molecular weight did not affect the characteristics of collagen films.

The interaction of pre-polymerized silicic acid with collagen and gelatin is shown by increases in the film areas. The pH range in which these increases are detectable is greater when the concentration of the silicic acid is higher; with concentrations of 0·001 M or more the interaction can be demonstrated over a range of about pH 3 to 8. This range is the same as that observed for albumin and insulin. It seems probable that proteins generally will react with silicic acid in the pH range 3 to 8, although the reaction is not detectable by the experimental method used when the molecular size of the silicic acid is so small that the adsorbed molecules do not make contact when the film is compressed. Whilst prepolymerized silicic acid in the substrate tans films of albumin, insulin and methaemoglobin over only a narrow range in the region of pH 6, the collagen films are tanned over a much wider range, about pH 3 to 8. The tanning is shown by a decrease in the compressibility of the film, larger specific areas and solidification of the film indicated by a marked difference between the force/area curves relating to the first and the second compressions over the whole pH range in which interaction occurs.

The globular proteins which interact with silicic acid give coincident curves for the first and second compressions at all pH values except near pH 6. Silicic acid sols gel most readily in this pH range, and the alteration in the force/area curve on a second compression must be related to the polymerization of the adsorbed silicic acid. This is unlikely to hold for collagen over the whole range of pH 3 to 8, because the curves relating to the first and second compressions are not coincident even at pH values where concentrated silicic acid sols do not gel for hours. At these pH values at least, the effect must involve the cross-linking by adsorbed silicic acid of the polar parts of the folded collagen units. As we have seen, the polar sections remain in the aqueous phase on compression, and they may be brought sufficiently close for interaction to occur through polysilicic acid links.

There are then, two distinct processes either or both of which may be involved in the tanning of a protein film by silicic acid, namely, (i) the polymerization of the silicic acid which has reacted with the film and (ii) the bridging of sections of a folded molecule by silicic acid. Both processes, presumably, may also be involved when protein films are tanned by other polyhydroxy compounds. Moreover, the possibility of interaction and of tanning depends also on the condition of the protein film; a film may be tanned when it is under pressure even though the substrate is too dilute to produce an effect when the film is at zero pressure.

Gelatin, hide powder collagen and tendon collagen films resemble one another in that they give similar specific areas and they are all tanned over a wide pH range, but tendon collagen films show differences in detail. (i) The maximum areas are observed at pH 7 to 7·5 for tendon collagen instead of 6 to 6·5. (ii) The interaction of tendon collagen with silicic acid is shown by decreased rather than by increased areas, except at pH 7·0 to 7·5. (iii) The minimum concentration of silicic acid which is effective in altering the characteristics of the film is much higher for tendon collagen.

A very low concentration of silicic acid will alter the properties of films of hide powder collagen and gelatin. When the substrate contains only 0·0001 M silicic acid, interaction is just detectable. No interaction with the films of tendon collagen could be detected when the concentration of the silicic acid was below 0·001 M. The pH range over which interaction is observed depends on the concentration of the silicic acid; when this is 0·002 M, interaction can be detected between pH 3 and 8, but at the minimum effective concentration, interaction can only be detected at the pH which gives the greatest increase in film area.

The reasons for the differences in the behaviour of the tendon collagen and the gelatin are not clear but several factors may be involved. The compositions of the three materials are not identical. The hide powder collagen and the gelatin are degraded and somewhat de-aminated, consequently the isoelectric point is lower and the number of end-groups is larger. The solvents were different in each case and this inevitably alters the rate of spreading; if the spreading is sufficiently slow, interaction with silicic acid may occur before the process is complete. This could explain the observed reduction of the specific area for tendon collagen. Since the specific areas are greater in the region of the isoelectric point, it must be assumed that the rate of reaction is slow in this region, a reasonable assumption because the protein has no overall charge at this pH so that silicate ions will not be attracted to the film.

The fact that a higher concentration of silicic acid is required to tan a film of tendon collagen may be explained, again, either by the slower spreading rate, or by there being fewer reactive groups available in the less degraded collagen.

Certain curves suggest that there is a minor peak in the force/pH curve of tendon collagen films on polysilicic acid at pH 5·5. This is near the pH at which most proteins give a maximum, being the pH at which silicic acid gels most rapidly. The peak is so small that it may be due to experimental error. In any case it can serve only to emphasize that the polymerization of the adsorbed silicic acid is of

minor importance in the tanning of collagen films as compared with the inter- and intra-molecular linking of sites in the protein by silicic acid.

The mechanism of the formation of natural collagen fibres is being studied in several laboratories. Silicic acid is known to be effective *in vivo* in the production of collagen fibres in silicotic lesions. In the fibres of silicotic nodules, silica, probably as silicic acid, is associated with the collagen fibres and can be observed in histological sections.

Polysaccharides are normally associated with collagen in all connective tissue. Partridge [10] and Jackson [11] suggested that the acidic polysaccharides stabilized collagen by cross-linking but Fitton Jackson [12] questioned this. Meyer [13] suggested that the acidic polysaccharides may act as templates on which the collagen fibrils are orientated. Much of the polysaccharides occurring in connective tissue is not acidic,[14] however, and it seems possible that the polysaccharide may be produced initially as a complex with the procollagen to prevent the premature precipitation of collagen fibres.

The polysaccharide content of connective tissue is reduced as normal tissue ages, and this reduction is accompanied by a decrease in the solubility of the collagen. The solubility of all regular polyamides, e.g. Nylon-1·5, is very small but is increased by other substances with which hydrogen bonding can occur; the decrease in solubility with ageing may be a direct result of the removal of polysaccharide. Silicic acid may well cause fibre development by replacing the polysaccharide of the collagen + polysaccharide complex to form a less soluble collagen + silicic acid complex.

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