The Effect of Electric Current on Rat Tail Tendon Collagen in Solution*

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The effect of electric current on dilute acetic acid solutions of soluble collagen has been studied for impressed voltages of from 0 to 25 volts. Above 2.6 volts the formation of collagen bands (herein defined) were observed at times inversely proportional to the applied voltage. Band formation is attributed to the process of electrolysis. It has been shown that the high pH values are generated in the area of the cathode, and that they are sufficient to induce collagen to precipitate. The nature of the process is such that it cannot occur *in vivo* as a response to stress induced biopotentials. Reports in the literature describing the effect of implanted voltage sources are interpreted in terms of the mechanism described here.

Key words: Collagen — Electric current — Electrolysis — Precipitation — Bone.

L'effet du courant électrique sur la collagène soluble, en solution dans l'acide acétique dilué, a été étudié pour des voltages, variant de 0 à 25 volts. Au-dessus de 2,6 volts, la vormation des bandes collagéniques (définies dans ce travail) parait inversement proportionelle, dans le temps, au voltage appliqué. La formation des bandes parait liée au processus d'électrolyse. Les auteurs démontrent que les pH élevés se situent au niveau de la cathode et qu'ils sont suffisants pour induire une précipitation du collagène. Les résultats antérieurs, publiés dans la littérature, décrivant l'action de courant électrique implanté, sont interpretés en fonction du mécanisme étudié au cours de ce travail.

Die Wirkung eines elektrischen Stromes auf verdünnte essigsaure Lösungen von löslichem Kollagen wurde bei Spannungen zwischen 0 und 25 Volt untersucht. Über 2,6 Volt wurde die Bildung von Kollagenbanden (in der Arbeit näher beschrieben) beobachtet, und zwar nach Zeiten, die der angewandten Spannung entgegengesetzt proportional verliefen. Die Bandenbildung wird dem Elektrolyseprozeß zugeschrieben. Wir konnten zeigen, daß sich die hohen pH-Werte rund um die Kathode entwickelten und daß diese genügen, um die Kollagenfällung zu veranlassen. Die Natur dieses Vorganges ist solcher Art, daß er *in vivo* als Antwort auf durch Stress verursachte Biopotentiale nicht vorkommen kann. Der hier beschriebene Mechanismus erlaubt es, Literaturangaben über den Effekt von implantierten Spannungsquellen zu interpretieren.

Introduction

Stress-induced biopotentials have been reported in a number of collagenous tissues (Fukada and Yasuda, 1957; Bassett and Becker, 1962; Fukada and Yasuda, 1964; Braden *et al.*, 1966; Cochran *et al.*, 1968). Their origin, and role if any, in the growth process are still being debated (Becker *et al.*, 1964; Shamos and Lavine, 1964; Jahn, 1968; Bassett, 1968). The measured biopotentials are

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of the order of several millivolts and it has been postulated that they may affect growth by altering cellular activity and/or by influencing the orientation and aggregation of tropocollagen molecules (Becker *et al.*, 1964; Bassett, 1968).

Evidence in support of the latter possibility was first described by Becker *et al.* (1964). They found that if a solution of rat tail tendon collagen was subjected to a D. C. voltage of about three, an opaque zone or band would form in the vicinity of the cathode. When the band was "fixed" by the addition of sodium chloride, and stained, fibers were found running parallel to the long axis of the band. Since the current (1 μa) was within the physiological range, the possibility was advanced that fibrils could be orientated *in vivo* by a similar mechanism as a response to the stress-induced biopotentials. This idea received further support in 1966 when Bassett observed collagen band formation for lower values of the current and voltage (0.04 μa, 130 mV) and in 1968 when it was shown the salt-fixed band contained fibers which exhibited the typical axial period of native collagen (Bassett, 1968).

The collagen bands occur for low voltages and at a finite distance away from the cathode, two facts which tend to rule out an electrophoretic mechanism. Furthermore, the geometry of the formed band is such that it is at right angles with the direction of current flow, suggesting some directionally dependent interaction. Beyond these preliminary considerations, the reason the band forms is unknown.

In view of the possible importance of the phenomena in the previously proposed bone growth control system (Becker *et al.*, 1964) we have undertaken a study to determine the responsible mechanism. Our aim is threefold; to determine (a) the mechanism of band formation *in vitro*, (b) the possibility of the process occurring *in vivo* as a response to the stress-induced biopotentials, (c) the possibility of the process occurring *in vivo* as a response to other sources of voltage.

Materials and Methods

Tendon from the tail of adult rats was extracted for 24 h with 0.1 M acetic acid at 4°. The solution was clarified by centrifugation and filtered through coarse, medium and fine sintered glass. It was then diluted 24:1 with pure (distilled-deionized) water and dialyzed at 4° for 72 h against repeated changes of pure water. This stock solution has a pH of 5.1, and a concentration of less than 0.01%; it was maintained at 4°, and all experiments were performed at room temperature. An aliquot of solution was prepared for study by warming to room temperature for 30 min, and then filtering through 0.8 μ Millipore filters.

Small chambers were used to contain the solution during passage of current. They were constructed of Plexiglass, principally with stainless steel electrodes, since they give results identical to those obtained using platinum electrodes. Two basic electrode configurations were employed. In one, the solution reservoir was formed by gluing a 0.25″ = high cross-section of tubular Plexiglass to a Plexiglass base. The electrodes, which were 10 mil wire, contacted the solution through holes oppositely located in the wall of the cross-section. This arrangement resulted in a non-uniform electric field in the solution. In the second, the electrodes were 0.25″ = high parallel metal plates with the two remaining sides of the solution reservoir and the base being Plexiglass. This arrangement gave a uniform electric field. In both cases, the electrode separation was 0.5″.

After final filtering the solution was pipetted into the chamber reservoir. Current was supplied by variable D. C. power supply and monitored continuously with a microammeter. Band formation in the chamber was observed with trans-illumination against a black felt background by means of a dissecting microscope of 7× to 40× magnification.

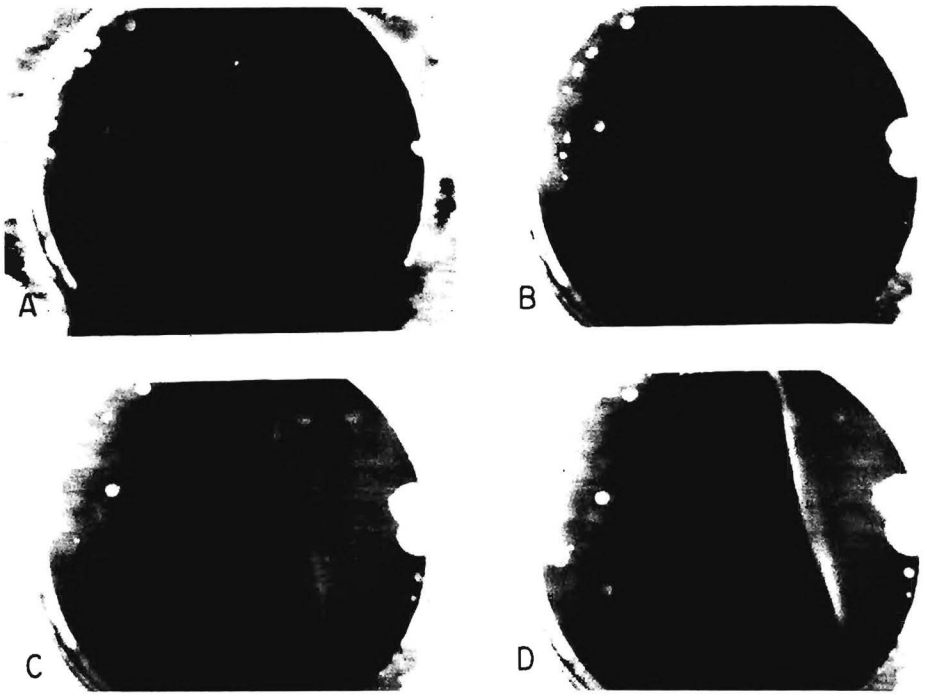

Fig. 1. Effect of passing current through a dilute solution of acid-soluble collagen. *A* $t = 0$; *B* 1 min; *C* 3 min; *D* 8 min. At 10 volts maximum current 65 μa. Chamber diameter, 0.5 inch. Electrodes along the horizontal, cathode at right. Exposure time 8 sec

Results

The effect of passing a current through a dilute solution of acid-soluble collagen is shown in time sequence in Fig. 1. Initially the chamber is clear. At some time after current flow is initiated a uniformly opaque zone forms at the cathode side of the chamber (Fig. 1, *B*). Subsequently the area of the opaque zone decreases and its anode edge becomes the dominant observable feature (Fig. 1, *C*, *D*). When viewed through the dissecting microscope, without the benefit of time exposure photography, only the anode edge of the zone can be clearly seen. It is this edge which we call the "band". The band routinely forms with a slight concavity toward the cathode thus appearing to reflect the electrical symmetry of the chamber. When the chambers with parallel plate electrodes were used, a sequence similar to Fig. 1 was seen. Band formation was parallel to the cathode and some distance away. This observation demonstrated that electric field non-uniformity was not a necessary factor in the mechanism of band formation, and so the remaining experiments were carried out with the more convenient wire electrode chambers.

The time required for band formation is inversely dependent on the applied voltage. This is illustrated in Fig. 2 for voltages greater than five. For smaller voltages the formation time becomes very long. For instance, at 2.6 volts, about 24 hours are required. We have been consistently unable to observe the phenomenon at voltages lower than 2.0 volts for observation times as long as 48 hours.

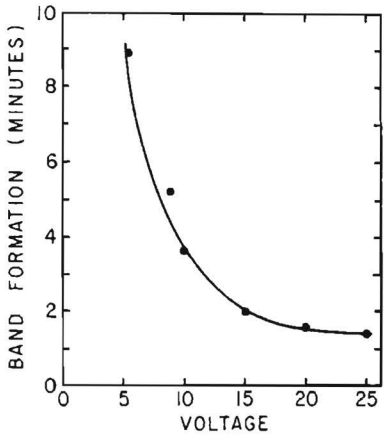

Fig. 2. Time of initial visualization of the band as a function of the applied voltage

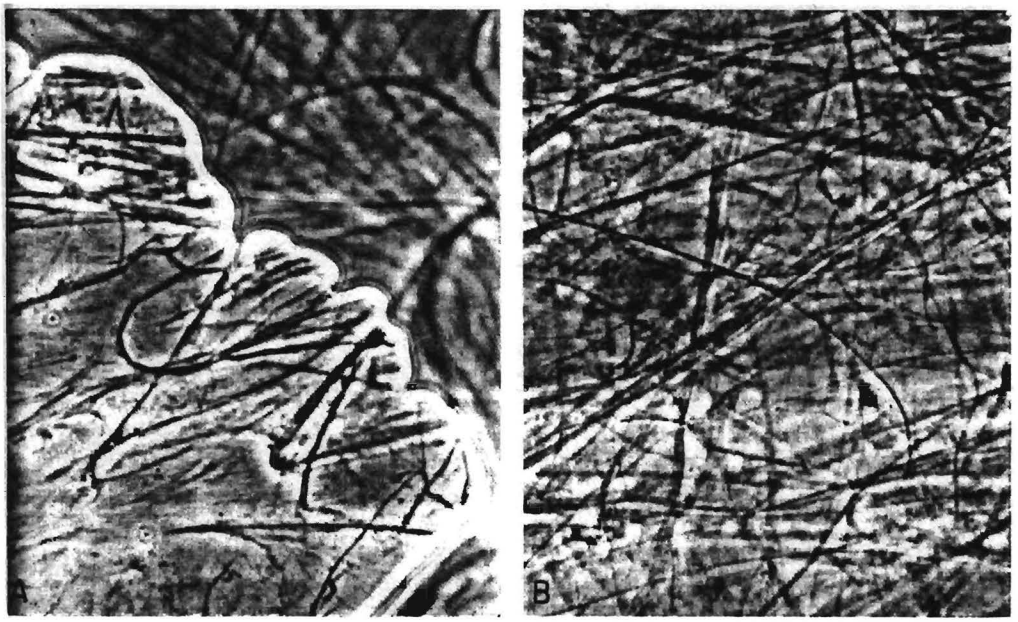

Fig. 3. Light microscope appearance of the band phase, ×400. Formed at 10 volts. The band was removed from solution, placed on a slide, covered with a cover slip, and photographed immediately without fixation or staining. The fibrous appearance is evident

Once formed, the band will dissolve in about two hours after the current is stopped; band formation can then be exhibited again in the same solution. The band will dissolve in distilled water in about twelve hours. Heating a solution to 40° for one hour is sufficient to destroy its ability to show band formation.

The band possesses sufficient mechanical strength to allow its removal from the solution with forceps, without the addition of any chemical substance. Fig. 3 illustrates the light microscope appearance of a band recovered in this manner.

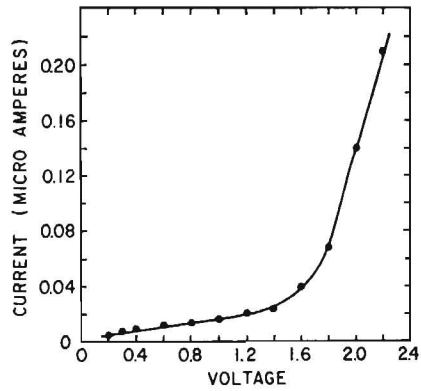

Fig. 4. Current versus voltage for collagen solution studied

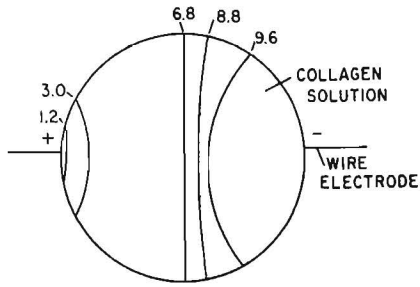

Fig. 5. Iso-pH lines in a collagen solution between wire electrodes at 10 volts. Lines were determined by observing the positions of the color transitions of the water soluble sodium salts of Cresol Red, Bromo Phenol Blue, Bromocresol Purple, and Thymol Blue. Between parallel metal plate electrodes, the iso-pH lines are parallel to the electrodes

Routinely, we observe this fiber-like feltwork, with no evidence of any preferred orientation. Histological slides of the band appeared amorphous and exhibited less structure. Electron micrographs of fixed, embedded and stained bands were generally structureless, although for some fixitives fibers were observed. In no case did we observe the 640 Å axial repeat period typical of normal collagen.

The apparent threshold for the effect in the vicinity of 2 volts led us to study the voltage-current characteristics of the solution in this region. The results are given in Fig. 4. The sharp increase in current at about 1.7 volts is a result of the onset of electrolysis of the solution. We now present evidence to show that electrolysis is responsible for the effect of band formation observed here.

Electrolysis of most dilute aqueous solutions between inert electrodes occurs at about 1.7 volts (Lingane, 1953). The electrode reactions (Glasstone, 1942; Lingane, 1953) are such that if the solution is not buffered, or vigorously stirred, the cathode area will become basic and the anode area acidic. While there is no net change in pH, there is a pH gradient during the time the solution is electrolyzed. The extent of the gradient in the collagen solution was measured by adding pH indicators and observing the color changes which occurred. The individual indicators have an effect of the order of 10% on the ambient pH, so that this procedure is only approximate. Fig. 5 is the summation of the results

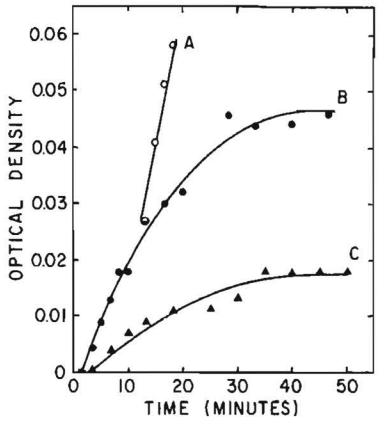

Fig. 6. Increase in optical density at 400 mμ as a function of time. Initial pH of collagen solution was 5.1. At $t = 0$, pH was adjusted by the addition of NaOH: *A* to 9.8; *B* to 9.6; *C* to 9.4. The subsequent increase in optical density is a measure of the rate of formation of a gel-like precipitate. The ionic strength due to the NaOH was not larger than 0.001

of several such experiments using different indicators. It is a measure of the pH profile which exists in the chamber about 3 minutes after electrolysis is begun at 10 volts. Band formation occurs in the region of the chamber at which the pH becomes greater than about 8.5, and invariably after the pH gradient is created.

It is well known that collagen can be precipitated from solution by altering the pH, temperature or ionic strength within certain limits (Wood, 1964). It remained then to show that these limits were encompassed by the chemical conditions present at the site of band formation, which were constant temperature, essentially zero ionic strength and high pH. We simulated these conditions by adding dilute sodium hydroxide to the collagen solution and examined for precipitation by measuring the optical density. The result, given in Fig. 6, shows an increase in optical density which accompanies the formation of a gel-like precipitate, indicating that the chemical conditions at the band site are conducive to precipitation.

If band formation is a result of the high pH generated by electrolysis, one expects no effect with alternating current. Such is the case for the frequency range studied (10^3 to 10^{-1} Hz).

Subsequent to its initial visualization, the growth in mass of the band is aided by some combination of electrophoresis and diffusion. When the chamber is divided vertically into two roughly equal sections by a filter sufficient to block migration of the collagen monomer (tropocollagen), the mass of the resulting band is reduced.

An effect related to band formation in collagen solutions has been observed in 0.05% solutions of gelatin derived from pig skin. The effect manifests itself as a clouding of the solution at the cathode side of the chamber. The gelatin does not aggregate and hence cannot be removed mechanically. The opaque formation diffuses in a few minutes after the current is stopped, and re-forms upon initiation of the current.

Discussion

The formation of a band (herein defined) occurs in dilute solutions of soluble collagen for D. C. voltages greater than 2.6 at room temperature. We have shown that a pH gradient is generated by the accompanying electrolysis and that the pH values thus achieved are sufficient to induce collagen to precipitate at a rate dependent on the particular pH. The gross configuration the band assumes as it forms is determined by the symmetry of the diffusion of hydrogen and hydroxyl ions which are generated at the electrodes. The subsequent changes that occur are shown in Fig. 1. Electrolysis begins at about 1.7 volts, and above 2.6 volts the pH gradient produced is sufficiently steep to permit band formation. We have observed no other effects at lower voltages.

The wet, unfixed, unstained band exhibited a fibrous appearence in the light microscope under phase contrast; samples fixed and stained for the light microscope and the electron microscope generally showed no structure. The solubility of the band in distilled water indicates that the aggregation of tropocollagen molecules have relatively weak cross-links. We believe that this "loose" structure was disrupted by the chemical action of the fixitives and stains employed, accounting for the absence of microscopic detail. Whatever the nature of the tropocollagen aggregate, its ability to form is a property of the undenatured molecule, since tropocollagen solution heated to 40° will not exhibit any effect, and more concentrated solutions of animal skin gelatin become opaque in the cathode area but do not aggregate.

Previous reports (Becker *et al.*, 1964; Bassett, 1966; Bassett, 1968) have described preferential alignment of fibers in small areas. We find such areas, but for our methods of band recovery and observation they are randomly distributed, so that no overall "grain" is discernable (Fig. 3). For the electric fields employed here, and previously (Becker *et al.*, 1964; Bassett, 1966, 1968), any preferred orientation of tropocollagen in solution will be extremely small (Yoshidka and O'Konski, 1966). If precipitation of tropocollagen is initiated at specific nucleation centers as envisioned by Wood (1964), one expects a random orientation of such centers such as we observe. At higher electric fields, the tropocollagen molecules in dilute acid solution become aligned along the direction of the field (Yoshidka and O'Konski, 1966).

In view of the nature of the mechanism responsible, we conclude that it probably cannot occur *in vivo* as a response to the stress-induced biopotentials. They are about two orders of magnitude too small to affect the polymerization and precipitation of collagen from solution by this method. They are many more times too small to produce a preferential orientation of tropocollagen in solution (Yoshidka and O'Konski, 1966). The possibility remains of other effects due to the biopotentials. If so, they must be restricted to a more physiological situation, since we observe no effects in dilute acid solutions for impressed voltages of the magnitude of the bone stress potential (1 mV).

The electrolytic effect may occur *in vivo* as a response to other sources of voltage. Potentials of several hundred millivolts are found in living cells, and higher voltages of biological origin have been reported in certain aquatic forms. In addition, the electrolytic properties of physiological fluid are unknown.

While decomposition voltages for aqueous solutions are commonly one to two volts, they are known to depend on many factors such as concentration, temperature, and electrode material. The decomposition voltage of physiological fluids, and the extent and efficiency of buffering by inorganic ions have not been reported. Thus, the possible application to naturally occurring processes remains unexplored.

An additional possibility is the occurrence of the effect as a response to an external voltage applied to an organism. The literature contains at least three *in vivo* studies describing the effect of an external voltage applied across the femora of laboratory animals (Bassett *et al.*, 1964; Minkin *et al.*, 1968; Lavine *et al.*, 1968). All three noted enhaced bone growth. In particular, Bassett *et al.* (1964) reported that one effect of the applied voltage was an increased number of young mesenchymal cells and osteoblasts. An additional effect may have been the precipitation of mineralizable collagen by a mechanism similar to that described here. This interpretation is supported by the facts that (a) gas evolution was reported, indicative of some electrolytic process, (b) growth was in the vicinity of the cathode and (c) the reported growth exhibited a voltage threshold large enough to be conceivably attributed to the onset of electrolysis. No growth was observed when the potential between the implanted electrodes was 0.5 volts, while growth was noted for potentials of about 1.1 and 1.4 volts. Thus, three salient aspects of that experiment support the interpretation given above.

In conclusion, the bone stress potentials are too small to produce orientation or aggregation of tropocollagen in solution. If the potentials are related to growth, they act through some other mechanism.

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