EXPERIMENTS CONCERNING THE EFFECT OF ENZYMES ON THE RECONSTITUTION OF COLLAGENOUS FIBRILS IN VITRO

A Preliminary Report*

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Existence and nature of the cement substance which binds the collagenous fibers and fibrils have been widely discussed. It has been shown that this material, probably mucoproteins, can be, at least partially, removed from the fibers not only by salts such as KCl and $CaCl_2$, but also by treating the fibers with trypsin (13). A cement substance which is probably of a nature similar to that within the microscopically visible fibers has been shown by the electron microscope to be present between the cross-banded microfibrils (Wassermann (13)).

Considering the coexistence of mucopolysaccharides and collagenous material during fibrillogenesis, it may be asked whether or not mucopolysaccharides take part in the building and the structure of the collagenous microfibrils. This question has been raised repeatedly, and several authors using chemical and physicochemical methods came to the conclusion that a collagen-mucopolysaccharide complex does exist (Burton *et al.* (1), Hall *et al.* (4), Loeven (7), and Tustanovskii *et al.* (11)).[1] S. F. Jackson and R. H. Smith (6) suggest that "the mucopolysaccharide and protein component of the granules (of the fibroblasts) may have fibrogenic properties." On the other hand, Schmitt, Gross, and Highberger (10), considering the question "whether or not a small amount of carbohydrate material is associated with the tropocollagen in forming collagen fibers," found by their own analysis "that the most highly purified collagen contains only a very small carbohydrate residue," which may be only an impurity due to adhering ground substance material. The observation that trypsin seems to hydrolyze the cement substance may offer an interpretation of Sizer's statement (9) that collagen fibers are, contrary to the experience of the histologists, digestible in trypsin when cut in very small pieces, and of D. S. Jackson's suggestion (5) that chondroitin sulfuric acid may serve as "a factor in the stability" of collagenous fibers: that is, not the collagen but the mucoprotein cement substance might have been attacked by trypsin in Sizer's, or by hyaluronidase in Jackson's experiments. On the

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[1] Since this report was written, a paper by W. Grassmann and K. Kuhn (*Z. physiol. Chem.*, 1955, **1**, 16) has been received. The authors found that collagen and "procollagen" undergo degradation when dissolved by periodate and phenyliodoso acetate, and concluded that "the degradation is probably based on an attack on the carbohydrate groupings present in collagen and procollagen."

other hand, the existence of a collagen-mucopolysaccharide complex remains to be considered. Instead of entering a collagen-mucopolysaccharide "complex," mucopolysaccharides might participate in the building of microfibrils as a kind of cement substance in between subunits of the fibrils. This suggestion has been derived from observations concerning "primary fibrils" as intermediate building blocks in fibrillogenesis (Wassermann (12)).

An attempt to approach this problem by a combination of enzyme treatment and reconstitution of microfibrils *in vitro* was based on the following considerations. If hyaluronidase action prevented the reconstitution of regular fibrils this would indicate a participation of mucopolysaccharides in their formation. An effect of trypsin would be due either to a direct effect on collagen, as suggested by Sizer, or to a degradation of mucoproteins serving as binding material of subunits of the microfibril. The effect of trypsin on collagen may be compared with that of pepsin, which has a similar but potentially more drastic effect.

EXPERIMENTAL

Aqueous solutions of pepsin,[2] trypsin,[3] or hyaluronidase[4] were added to acidified collagen solutions (prepared from rat tail tendon) in the manner described by Porter and Vanamee (8) at enzyme concentrations of 0.1 and 0.25 per cent. The pH values in the different experimental solutions ranged from 3 to 7. After incubation at 39°C. for about 20 hours, reconstitution was accomplished usually by raising the pH to 5.8; in those cases in which pH was already at or above 5.8, reconstitution in the presence of the enzymes began during the incubation period.

The reconstructed structures from solutions treated with the different enzymes exhibited marked differences in appearance in the electron microscopic preparations.

The inhibition in reconstitution of cross-banded fibrils resulting from pepsin action (Fig. 1) was variable, apparently depending on the degree of hydrolysis produced.

In the majority of our experiments with hyaluronidase, the only fibrils resulting from the reconstitution were tortuous and rope-like structures (Fig. 2), composed of short, often tactoidal parts which were helically entwined but not tightly packed. Although the exposure of control collagen solutions to a temperature of 39°C. for 20 hours appears to retard the reconstitution, fibrils similar to those appearing in the hyaluronidase-treated solutions did not occur. Electron micrographs of the dried residue of hyaluronidase alone revealed no structures resembling the rope-like fibrils or their components.

The effect of trypsin on the reconstitution remains questionable at present. Gross (2, 3) has demonstrated that Armour's crystallized trypsin itself ex-

[2] Pepsin, crystallized (Armour Laboratories).

[3] Trypsin, crystallized (Armour Laboratories).

[4] Hyaluronidase (Nutritional Biochemical Corporation).

hibits fibrous structures under certain conditions. We recognize, therefore, that the appearance of fibrils of this kind in the preparations used in the present work does not allow a decision regarding the effect of trypsin on the reconstitution. The final decision will depend on the separation of the interfering filaments from the true collagen structures, either spatially by way of diluting the preparations or by differential centrifugation. The latter procedure has been used in experiments where, following Sizer's suggestion, fibers from the rat tail tendon were teased and cut and then incubated with trypsin at pH 7.8 and 39°C. Most of the questionable material could be removed by repeated centrifugation and washing, so that in the electron microscopic preparations from the remaining material, the collagenous structures could be demonstrated. The isolated and fraying collagenous fibrils and their components which occur after trypsin treatment must be compared to the corresponding degradation products which appear when similarly prepared collagen fibers are incubated at pH 7.8 with saline alone. Only significant differences may be accepted as evidence for an effect of trypsin. The same separating technique can be applied after the reconstitution of fibrils from trypsin-treated collagen solutions.

So far, Jackson's suggestion that mucopolysaccharides are a stabilizing factor in collagen structures seems to be supported to a certain degree by our experiments with hyaluronidase. Sizer's report of the digestibility of collagen by trypsin is at present still unconfirmed by electron microscopic investigation.

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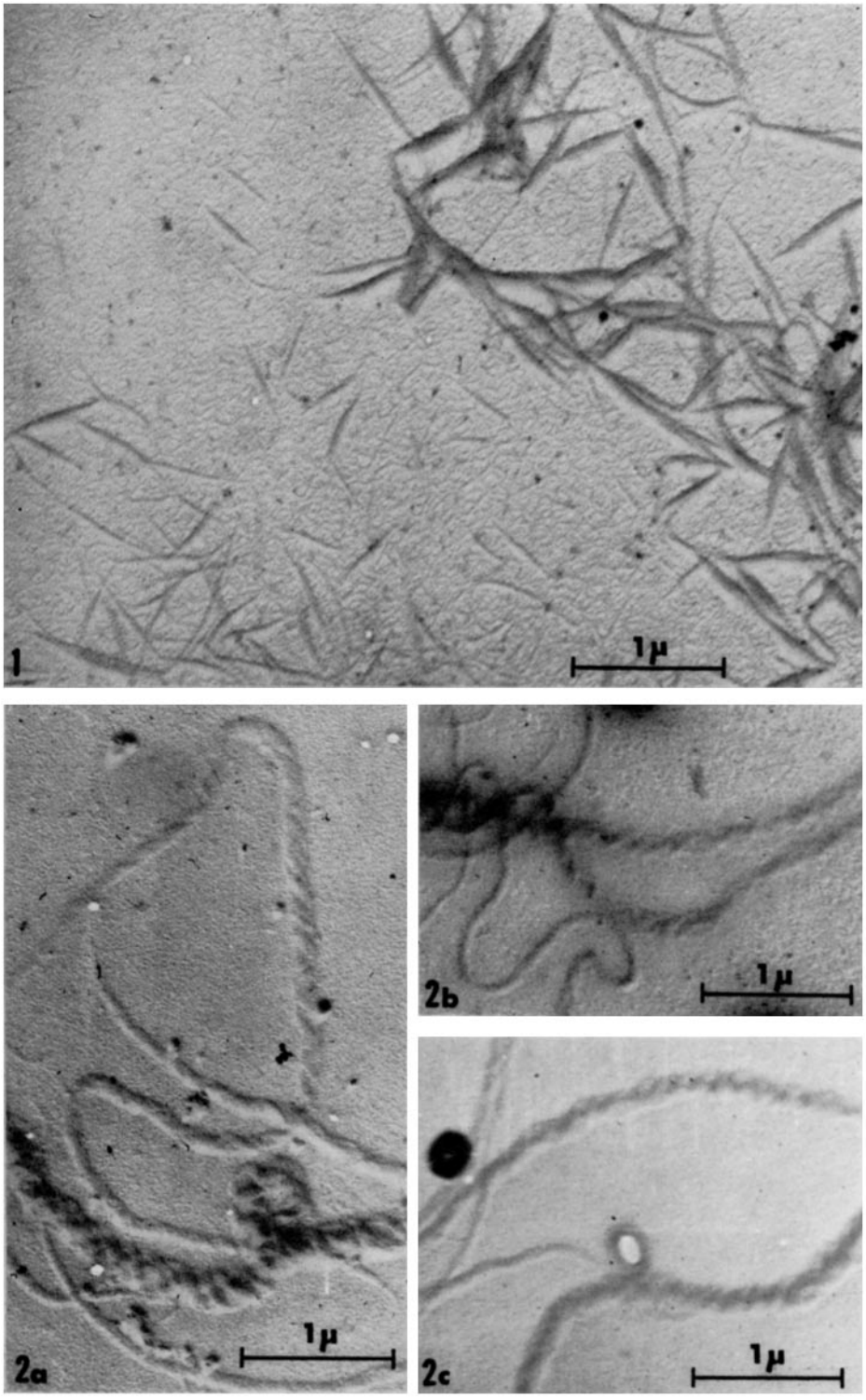
EXPLANATION OF PLATE 95

FIG. 1. Electron micrograph of reconstruction products as they appear after incubation of acid collagen solution at 39°C. for 20 hours with 0.25 per cent pepsin. The formation of fibrils was not completely prevented as in other experiments of this kind. Small particles fill the background and tactoidal bodies of various sizes have formed. In some of them, a periodic structure along the axis is indicated. × 21,000.

FIG. 2 Electron micrograph showing the rope-like tortuous fibrils derived from collagen solution after incubation with 0.5 per cent hyaluronidase at 39°C. for 20 hours. *a*, *b*, *c* are from three different experiments. × 21,000.



(Wassermann and Lindenbaum: Enzyme effect on collagenous fibrils)