LETTERS TO THE EDITOR

A DIRECT EVIDENCE FOR THE NON-EXISTENCE OF ESTER-BONDS IN COLLAGEN CHAINS

Introduction

The sub-unit structure model of collagen chain with ester-bonds between the sub-units was earlier suggested by Gallop et al., on the basis of their findings on the reaction of hydroxylamine and hydrazine with denatured collagen. This model was once considered as an acceptable one for collagen and the results in many laboratories were explained in view of this model. In an earlier communication we have reported that the soluble fraction obtained by treating shark-fin rays (elastodin) with formic acid consists of smaller sub-units having sedimentation coefficient ($S_{20, w}$) value of 1-6 compared to 3-2 for an intact single collagen chain. As formic acid under such experimental conditions does not affect peptide bonds, the observation was explained by assuming that shark-fin collagen chains are built up of smaller sub-units linked together by non-peptidic bonds, possibly of ester-type which might be broken by formic acid treatment. Possible mechanisms of formation of such ester-bonds in vivo in the growing polypeptide chain were also suggested. But later, another group of workers, particularly Bornstein et al., claimed that the sub-units which were obtained by treating collagen with hydroxylamine and hydrazine are not linked through ester-bonds but through cyclic imides formed by the condensation of the side-chain amide group of asparagine with amide nitrogen of the subsequent glycine residue in the chain. Both these groups, however, arrived at their own conclusions through indirect approaches. No direct proof for the presence or absence of ester-bonds in collagen chains has hitherto been offered. Considering that a study on the action of esterase on collagen chain should provide us, with a direct evidence regarding this, it was thought worthwhile to investigate the problem using a liver esterase as a probe. Liver esterases appear to have macromolecular substrate specificity also since these enzymes are considered to be reactive towards the ester-bond in the aminoacyl-RNA complex during the protein synthesis in liver.

Experimental

Preparation of Collagen Chain.—The swim-bladder removed immediately after slaughtering the carp was cleaned and cut into small pieces. These were then treated with 0-1% acetic acid for 24 h, after which the solution was filtered through glass wool. All operations were performed at 4° C. The filtrate was lyophilized and stored at 4° C in a desiccator. The a 1-chain was isolated from thermally (40° C for half an hour) denatured collagen by chromatography on CM-cellulose. The desired fractions were pooled, dialysed and lyophilized.

Preparation and Purification of the Esterase.—Esterase was extracted from rat liver according to Okuda and Fuji and treated with pancreatic lipase (Calbiochem) and purified by gel filtration on Sephadex G-200 (Pharmacia) as described by these authors.

Esterase Activity.—Two methods were employed to check esterase activity, a spectrophotometric method using p-nitrophenyl acetate (pNPA) as substrate and a titrimetric method using methyl butyrate as substrate. In the former method the enzyme prepared had a specific activity 320 ± 10 μ moles/mg protein/h at pH 7 and 25° C, whilst against methylbutyrate the specific activity was 250 ± 10 μ moles/mg protein/h at pH 8 and 25° C.

Proteolytic activity.—Proteolytic activity of the esterase was checked by using the method of Levisohn and Aronson. To 12.5 mg portions of AZOCOLL (Calbiochem) suspended in 1 ml of 0.2 M sodium phosphate buffer (pH 7.6) 0.5 ml of the enzyme in the same buffer was added and the volume was brought to 2.5 ml with water. After incubation at 37° C for 15 minutes the suspension was filtered through Whatman No. 1 filter-paper. The absorbancy at 580 nM of the filtrate was measured in the spectrophotometer, Unicam, Sp-600.

Reaction of esterase with collagen.—Purified a 1-chain of Carp swim-bladder was digested with esterase at 25° C and also at 40° C for 15 hours at pH 8.0 with the collagen : esterase ratio 100:1. A pH-meter autotitrator was used to follow up the reaction.

Ultracentrifugation.—The sedimentation coefficient values of both enzyme-treated as well as untreated material were determined at 25° C using the Spinco Model E (Beckman) ultracentrifuge. The concentrations of the protein in phos-phot buffer (pH = 7, μ = 0.1) were kept the same (0.8%) in both cases.
Results and Discussion

Results summarised in Table I indicate that no hydrolysis of the collagen has occurred by treatment with the esterase. The $S_{20, w}$ value of 2.83 for the esterase-treated collagen was found to be almost the same as that (2.81) of the untreated one. These observations offer a direct evidence to support the view that no conventional ester-bonds are present in the collagen chain.

Table I

<table>
<thead>
<tr>
<th>Reaction of esterase with different substrates and with collagen</th>
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<tr>
<td>Reactions</td>
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<tr>
<td>1. Proteolytic substrates</td>
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<tr>
<td>AZOCOLL</td>
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<td>2. Ester substrates</td>
</tr>
<tr>
<td>$p$-Nitrophenyl acetoacetate (pNPA)</td>
</tr>
<tr>
<td>Methyl Butyrate</td>
</tr>
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<td>3. Collagen</td>
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<td>Purified el-chain of Carp swim-bladder</td>
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Our grateful thanks are due to Professor A. Sen, Bose Institute, for allowing us the use of some of his equipments.

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2. —, Ibid., 1966, 209, 73.

A Relation between Magnetic Susceptibility and Electronic Polarizability of Noble Gases

The molar diamagnetic susceptibility of a monatomic gas is given by the well-known expression

$$x_m = -\frac{N_e \mu}{6 \pi c^2} \sum \frac{\langle r_i^2 \rangle}{i}$$

(1)

where $\langle r_i^2 \rangle$ signifies the mean square radius for the $i$-th electron and the summation extends over all the electrons of the atom. Thus the theoretical calculation of diamagnetic susceptibility for any monatomic gas reduces to the evaluation of the integral

$$\langle r^2 \rangle = \int \psi^* r^2 \psi \, d\tau$$

(2)

where $\psi$ is the wave function for the normal state in zero field. Wave functions obtained by analytical and other approximations are usually employed in the calculation of $x_m$ since, except for hydrogen, which is not diamagnetic, the wave functions are not known exactly.

Expression (1) shows that the value of $x_m$ is dependent essentially on the effective size of the charge distribution within the atom. When the charge distribution is spherically symmetric, one may assume that the magnitude of the susceptibility is determined by an effective charge distribution having an average radius. In this case the experimental values of diamagnetic susceptibility for monatomic gases may be considered as a measure of the size of the charge distribution, in so far as the response of the electron cloud to a magnetic field is concerned.