THE INTERPRETATION OF ELECTRON MICROGRAPHS OF NEGATIVELY STAINED NATIVE COLLAGEN

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SUMMARY
Deamination of native rat tail collagen followed by treatment with 1,2-cyclohexanedione removes the positive charge from the side chains of the lysine and hydroxylysine residues and blocks approximately 70% of the positively charged side chains of the arginine residues. When such specimens are examined in the electron microscope after negative staining with 2% potassium phosphotungstate at pH 6.8-7.4 no change from the appearance of normal collagen is detectable. These findings suggest that electrostatic attraction does not occur between the stain and the side chains of the lysine, hydroxylysine and arginine residues of the collagen and justify the view that, at least with this stain and under these conditions, the effect is that of pure negative staining and not that of a mixture of positive and negative staining. Furthermore, the experiments indicate that possible electrostatic attractions between the positively charged side chains of lysine, hydroxylysine and arginine and the negatively charged side chains of residues such as aspartic acid and glutamic acid are relatively unimportant in the maintenance of the light cross- striations of negatively stained collagen. However, the loss of these light cross-striations after treatment of collagen with urea implies that they are mainly associated with intermolecular hydrophobic and, possibly, hydrogen bonding.

INTRODUCTION
Collagen may be negatively stained for electron microscopy with 2% potassium phosphotungstate at pH 6.8-7.4. The stain should surround the specimen with electron-dense material and there should be no reaction between stain and specimen (Horne, 1965). This is the opposite of positive staining where such a reaction is desirable in order that the heavy atoms of the stain may be actively bound to the specimen and thus increase its contrast, any uncombined stain being washed off before examination in the electron microscope. Phosphotungstic acid at pH 4.2 has been used as a specific positive stain for collagen and is said to indicate the position of the polar groups, lysine and arginine (Hodge & Schmitt, 1960). Theoretically, the conditions for the negative staining of collagen appear to be satisfied by the use of potassium phosphotungstate at neutral pH. However, there has always been some doubt on this subject and it has been suggested that electrostatic attraction occurs between the neutralized heavy metal stain and the collagen and that the overall image seen in the microscope is a composite of both positive and negative staining characteristics (Hodge & Petruska, 1963).

Negative staining of native collagen with potassium phosphotungstate produces
alternate, broad, mainly light (A) and mainly dark (B) bands with a period of approximately 64 nm along the length of the fibril (Fig. 1). The A bands themselves are subdivided into narrower light cross-striations while other light cross-striations occur in the B bands (Cox, Grant & Horne, 1967). Treatment of the native fibrils with reagents such as glutaraldehyde and benzoquinone alters the image seen in the electron microscope after negative staining, the main A band being increased in width and a further light cross-striation becoming visible in the B band (Grant, Cox & Horne, 1967; Grant, Horne & Cox, 1965). Physical and chemical tests (Bowes & Cater, 1964, 1968) now demonstrate that the tropocollagen macromolecules are firmly cross-linked by covalent bonds, so that the increase in the light areas seen with negative staining may be attributed to their presence. The intermolecular cross-links are believed to prevent the penetration of the negative stain in the affected regions. On the basis of experiments such as these it was suggested that the light striations seen in the native collagen fibrils might also result from lateral bonding of the macromolecules. In the initial stages of fibril formation, before the appearance of covalent bonds that have arisen from the reaction of aldehyde groups derived from lysine, it is thought that the lateral bonds might be electrostatic, hydrogen and hydrophobic (Grant et al. 1965, 1967; Cox & Grant, 1969).

Experiments have been devised to decide (a) whether an electrostatic attraction occurs between the collagen and the potassium phosphotungstate at the neutral pH of negative staining and (b) the relative importance of the different lateral bonds for the maintenance of the light cross-striations seen in such negatively stained preparations of native collagen.

MATERIALS AND METHODS

Collagen preparations

Rat tail tendons were freshly dissected and washed in cold saline (0.9% NaCl).

Deamination

A solution of nitrous acid was prepared by mixing equal volumes of 1 M acetic acid (AR) and 1 M sodium nitrite (AR). Tendon fibres were treated for periods of 24 h and 5 days at room temperature.

Cyclohexanedione treatment

1,2-Cyclohexanedione has been used by Toi, Bynum, Norris & Itano (1965) to block the guanidino group of arginine. In the present experiments approximately 500 mg of tendon collagen were added to 10 ml of 0.5 M 1,2-cyclohexanedione in 0.1 M phosphate buffer at pH 7 and treatment continued for 5 days to 3 weeks at room temperature. In other instances the collagen was first deaminated before being treated with 1,2-cyclohexanedione.

Urea treatment

Urea in 0.03, 0.1, 0.5, 1, 3 and 6 M concentrations in 1% ammonium acetate at pH 7 was added to equal volumes of a suspension of washed rat tail tendon and left at room temperature for periods from 0.5-24 h.
Amino acid analysis

Dried samples of the deaminated, cyclohexanedione-treated, deaminated and cyclohexanedione-treated, and untreated native collagen were hydrolysed by heating with 6 N HCl for 6 h in sealed tubes in an autoclave at 123.4 kN m⁻² (15 lb in.⁻²) pressure. The hydrolysates were analysed by ion exchange chromatography using a Technicon amino acid analyser.

Electron microscopy

With the exception of the collagen treated with urea, treated and untreated collagen fibres were washed with either 0.1 M phosphate buffer or 1% ammonium acetate solution at pH 7 and ground with a small volume of the solution in an agate mortar and pestle. Fine suspensions of the material were negatively stained on grids with 2% potassium phosphotungstate at pH 6.8-7.4 (Brenner & Horne, 1959). Urea-treated preparations were not washed before negative staining. All preparations were examined in an AEI EM 6B/2 electron microscope at an accelerating voltage of 60 kV and at machine magnifications of 40,000 and 80,000. The microscope had been accurately calibrated using negatively stained beef liver catalase as a standard (Cox & Horne, 1968).

RESULTS

Amino acid analyses

After the treatment of collagen with nitrous acid for periods from 24 h to 5 days, 100% of the lysine and hydroxylysine had been deaminated. Treatments of collagen for both 5 days and 3 weeks with cyclohexanedione alone at pH 7 blocked approximately 70% of the arginine. In addition, the 5-day treatment decreased the amount of hydroxylysine by 35% and the amount of lysine by 20%, while the 3-week treatment reduced the hydroxylysine by 75% and the lysine by 45%. Deamination followed by cyclohexanedione treatment yielded specimens in which deamination of the lysine and hydroxylysine was complete and approximately 70% of the arginine was blocked.

Electron microscopy

Collagen that had been deaminated with nitrous acid for 24 h or 5 days was unchanged when compared with untreated native collagen (Grant, Cox & Kent, 1970). Moreover, collagen that had been deaminated and then treated with cyclohexanedione, also showed no change from native collagen (Figs. 1, 2). However, collagen that had been subjected to cyclohexanedione only, revealed a distinct light cross-striation in the B band. Collagen that had undergone treatment with urea showed definite changes, particularly with concentrations of 0.1 M urea and above. The changes ranged from complete loss of light cross-striations to a partial separation of the larger fibrils into much smaller fibrils that still retained some cross-striations (Figs. 3, 4).

DISCUSSION

Deamination with nitrous acid results in replacement of the e-amino group of the lysine and hydroxylysine side chains by a hydroxyl group. At neutral pH this does not carry a positive charge as would the e-amino group. Similarly, the blocking of the guanidino group of arginine by cyclohexanedione eliminates the positive charge that
is normally associated with the guanidino group at neutral pH. With positive staining of collagen at pH 4.2, ionic electron-dense compounds such as phosphotungstic acid are thought to be electrostatically bound to the positively charged side chains of the lysine and arginine of collagen. The washing of the specimen that occurs before examination in the electron microscope is thought to remove the lysine-bound stain, which is loosely attached, and not to remove the arginine-bound stain, which is firmly attached. Consequently, the latter is believed to be mainly responsible for the band intensities seen (Hodge & Schmitt, 1960). With negative staining at neutral pH no washing occurs after staining so that if electrostatic attraction did take place between the stain and collagen both the lysine and arginine might be expected to be involved. If then the image seen in the electron microscope were really a mixture of both positive and negative staining characteristics, some change would be anticipated when the positive charges on the lysine and hydroxylysine side chains were removed by deamination and 70% of the positively charged arginine side chains were blocked with cyclohexanedione. Since no change is seen it suggests that no positive-staining effect is involved when collagen is stained at pH 6.8–7.4 with 2% potassium phosphotungstate. The results thus justify the view that, at least with this stain and under these conditions, the effect is that of pure negative staining.

The decrease in the lysine and hydroxylysine after cyclohexanedione treatment alone and the appearance of a light cross-striation in the B band in the electron microscope indicate that some cross-linking may occur after this treatment. However, preliminary deamination effectively removes this ability of the cyclohexanedione to produce cross-links involving the lysine and hydroxylysine.

The amino acid analyses show that approximately 30% of the arginine remains unaffected by the cyclohexanedione. As the reaction product is stable to hydrolysis (Toi et al. 1965; Schroeder, 1968) it is reasonable to infer that this amount of arginine is inaccessible to cyclohexanedione. It may also be inaccessible to phosphotungstic acid.

The deamination and arginine blocking experiments indicate that possible electrostatic attractions between the positively charged side chains of lysine, hydroxylysine and arginine and the negatively charged side chains of residues such as aspartic acid and glutamic acid are relatively unimportant in the maintenance of the light cross-striations of negatively stained collagen. Consequently, electrostatic lateral bonding is presumably of limited importance in the maintenance of the structure of the collagen fibril at this stage.

The changes seen after the treatment of collagen with urea were similar to those described by Bailey & Tromans (1964). Urea has in the past been considered to be a hydrogen bond-breaking reagent (Tanford, 1961). However, more recent work has indicated that its main action may be in disrupting hydrophobic bonds (Tanford, 1968, 1970). Guanidine hydrochloride was also used but was found to react with the negative stains employed. Only 0.1 M guanidine hydrochloride with 2% ammonium molybdate as a negative stain yielded any usable preparations for electron microscopy. The changes were the same as those seen with urea.

It is concluded that the light cross-striations seen in preparations of native collagen
that have been negatively stained with potassium phosphotungstate are mainly associated with intermolecular hydrophobic and, possibly, hydrogen bonding. The part played by electrostatic intermolecular bonding appears relatively unimportant.

REFERENCES


(Received 2 August 1971)
All preparations have been negatively stained with potassium phosphotungstate at pH 6.8-7.4.

Fig. 1. Native rat tail collagen. Alternate, broad, mainly light (A) and mainly dark (B) bands with a period of approximately 64 nm are prominent. The A bands measure approximately 28 nm and the B bands approximately 36 nm.

Fig. 2. Native rat tail collagen that has been deaminated and then treated with cyclohexanedione. The appearances are unchanged from those of the untreated native fibril.
Negative staining of collagen
Fig. 3. Native rat tail collagen that has been treated with 0.5 M urea. The fibril is beginning to lose its light cross-striations.

Fig. 4. Native rat tail collagen after treatment with 0.5 M urea. The large fibril has separated into smaller fibrils that still retain some light cross-striations.