At zero time, various amounts of the enzyme preparation were added to the reference cuvette, thus allowing for a positive increase in the change in absorbance due to the oxidation of ascorbic acid. This change was recorded continuously at 265 mμ using a time-drive at 1 in./min. The rate was constant and could be extrapolated back to zero time.

The relationship between the amount of enzyme activity (change in absorbance per minute) and enzyme concentration can be seen in Fig. 2.

Absorbance results obtained spectrophotometrically were converted to establish enzyme units obtained manometrically (10 μl. oxygen/min)[2]. Temperature corrections were made on 10 μl. oxygen at 25° C and the corrected volume was converted to μmoles of molecular oxygen. One enzyme unit is equivalent to 0·405 μmole oxygen/min. Ascorbic acid requires only 1 atom of oxygen per molecule oxidized. Therefore, 0·81 μmole of $\frac{1}{2}$ oxygen/min is utilized per enzyme unit. From Daglich's results[2] ascorbic acid has an $E_{1\ cm}^{1\%}$ of 760 at 265 mμ. From this it was calculated that ascorbic acid has an absorbance of 4·4 per μmole in a 3-ml. volume. Therefore, 1 enzyme unit (0·81 μmole of $\frac{1}{2}$ oxygen/min) would be equivalent to an absorbance change of 3·58/min. Manometric and spectrophotometric assay of enzyme units of the same enzyme preparation gave 11 and 12 enzyme units/ml. respectively.

M. F. OBERBACHER
H. M. VINES

Florida Citrus Experiment Station
and Florida Citrus Commission,
Lake Alfred, Florida.

[1] Dawson, Charles R., and Magee, Richard J., *Methods of Enzymology*, 2, 831 (Academic Press, Inc., 1955).
[2] Daglich, C., *Biochem. J.*, 49, 635 (1951).
[3] Hewitt, E. J., and Dickes, G. J., *Biochem. J.*, 78, 384 (1961).
[4] Vines, H. M., and Oberbacher, M. F., submitted to *Plant Physiol.*

Presence of Phosphate-mediated Cross-linkages in Hard Tissue Collagens

IT has often been suggested that covalent interchain cross-linkages contribute to the stability of collagen fibrils[1,2]. Gustavson[1] speculated that ester bonds exist between free carboxyl groups and the hydroxyl groups of the hydroxy amino-acids. The work of Gallop et al.[3] and Hörmann et al.[4] has substantiated this proposal, implicating carbohydrate or sugar cross-linkage as well. We have found in our work with dentine collagen evidence for still another type of cross-linkage which might apply to the collagens of the calcified tissues, bone and dentine.

Bovine dentine collagen, decalcified with ethylenediamine tetraacetic acid (EDTA) at pH 7·5, was found to exhibit markedly different swelling and solubility characteristics in comparison with purified bovine corium collagen. For example, bovine corium collagen swells maximally to 700 per cent at pH 2·3 in salt free hydrochloric acid, whereas dentine collagen has no appreciable swelling in the entire acid-range. Bovine corium collagen can be rendered soluble to the extent of 8 per cent by isoionic heating at 60° C for 1 h. With bovine dentine collagen less than 2 per cent is solubilized under the same conditions after 20 h. These results obviously suggest a difference in the mode or extent of the intermolecular stabilization of these two types of collagen.

Comparative chemical analyses showed both collagens to have very similar amino-acid compositions and carbohydrate contents. However, the purified decalcified dentine collagen had a phosphorus content which, expressed in phosphate residues (HPO_3), averaged 0·4 per cent by weight. This phosphorus could not have been the result of residual calcium hydroxyapatite since the calcium/inorganic phosphorus ratio was 0·002 in the purified dentine collagen, compared with a ratio of 2·12 in the undecalcified dentine and 2·16 in pure calcium hydroxyapatite. Since the phosphorus could not be removed by prolonged EDTA extraction or dialysis, it is apparently present in a covalently bound form, presumably as the phosphate. The phosphate content noted here would correspond to 4 phosphate residues per 1,000 amino-acid residues, or to 12 potential cross-linkage sites per tropocollagen monomer unit. Phosphorus is essentially absent in the bovine corium collagens.

The phosphorus-containing moiety is present in a relatively stable form. On conversion of the purified, decalcified dentine collagen into gelatin by heating in water at 110° C overnight it was found that the non-dialysable gelatin still maintained two-thirds its original phosphorus content. Venom and spleen phosphodiesterases, used at their usual optimal pH, were ineffective in degrading the purified dentine collagen. The acid swelling and solubility properties of the treated collagen were unchanged after this treatment. However, these results certainly do not rule out the presence of phosphodiester linkages, since the highly organized collagen structure may mask the phosphate groups and diminish the enzyme activity. Likewise, the specificity of these enzymes may not be appropriate; all phosphodiesters are not equally good substrates for every phosphodiesterase[5]. In this regard it is pertinent to note that dentine collagen is also particularly resistant to the action of the usual protein denaturants (for example, 8 M urea) which have a strong denaturing action on bovine corium collagen. Work is in progress on the more accessible phosphate groups in the non-dialysable gelatins obtained by high-temperature hydrolysis. Investigations with oxidizing agents such as sodium periodate suggest that the phosphate may be associated with the carbohydrate moieties in dentine collagen.

These results lead us to suggest that the unusual properties of dentine collagen may be ascribed, in part, to the presence of cross-linkages mediated by phosphate groups. Perlmann[5] has described phosphate esters of three types in other proteins and peptides, —O—P—O—, —N—P—O—, and pyrophosphate, involving serine, threonine and arginine, and possibly carbohydrate. In addition, the hydroxy amino-acids peculiar to collagen, hydroxyproline and hydroxylysine should be considered as potential sites for phosphate ester formation.

ARTHUR VEIS
ROBERT J. SCHLUETER

Department of Biochemistry,
Northwestern University,
Medical School,
Chicago 11.

[1] Gustavson, K. H., *The Chemistry and Reactivity of Collagen*, 133 (Academic Press, New York, 1956).
[2] Veis, A., and Cohen, J., *J. Phys. Chem.*, 62, 459 (1958).
[3] Gallop, P. M., Seifter, S., and Meilman, E., *Nature*, 183, 1659 (1959).
[4] Hörmann, H., Riedel, A., Altenschöpfer, T., and Klenk, M., *Das Leder*, 12 175 (1961).
[5] Perlmann, G., *Advances in Protein Chemistry*, 10, 1 (Academic Press, New York, 1955).

Heterogeneity of Urinary Acid Mucopolysaccharides

687 mg of crude urinary acid mucopolysaccharides (preparation A) were precipitated from 30 litres of normal human urine according to methods previously described[1].

587 mg of A were incubated with crystalline papain at 37° for 70 h in phosphate buffer pH 6·30, 0·2 M, containing ethylenediamine tetraacetic acid 0·001 M and cysteine 0·005 M. The digest was applied to a 30 × 1 cm column of 'Dowex 1 × 2' (200–400 mesh, acetate form) and elution was performed with 200 ml. of acetate buffer pH 5·90, 0·1 M, followed by 200 ml. of 4 M sodium chloride. The