requirement that each comparison be between two proteins with the same total number of residues was relaxed to a requirement that the numbers do not differ by more than a few per cent.) In all there were 163 comparisons, of which seven showed significantly similar compositions. All of these seven showed significant sequence similarity when the sequences were examined. Of the remaining 156 comparisons for which significant composition similarity was not seen, 144 showed no significant similarity of sequences either. So the test gave the correct result in more than 90% of cases and, more important, in no case did it indicate similarity when none existed in the sequences.

To test the validity of $S\Delta n$ as an estimator of the number of sequence differences between a pair of related proteins, all possible pairs of known cytochrome $c$ sequences were examined. Averaged over 1830 comparisons, $S\Delta n$ was found to be slightly biased, estimating about 13% more sequence differences than there were in fact, with a coefficient of variation of 43%. Similar results were obtained with other sets of proteins, including insulin, the snake venoms, ribonuclease, crystallin and various globins. In all cases the observed behaviour of $S\Delta n$ was similar to that predicted.

Dayhoff, M. O. (1972) Atlas of Protein Sequence and Structure, vol. 5, National Biomedical Research Foundation, Silver Spring, MD
Dayhoff, M. O. (1973) Atlas of Protein Sequence and Structure, vol. 5, suppl. 1, National Biomedical Research Foundation, Silver Spring, MD

The 0-, 4- and 6-Sulphated Disaccharides of Chondroitin Sulphates: Their Electrophoretic Separation and Detection with $p$-Anisidine

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Chondroitin sulphates are depolymerized by chondroitin ABC lyase (EC 4.2.2.4) to yield unsaturated sulphated disaccharides 2-acetamido-2-deoxy-3-O-(β-D-glucuronic acid)-4(or 6)-O-sulpho-β-galactoses and from chondroitin 4-sulphate or chondroitin 6-sulphate respectively (Saito et al., 1968; Thurston et al., 1975). After separation by chromatography or electrophoresis, the sulphated disaccharides have been identified with alkaline AgNO$_3$ (Suzuki et al., 1968; Trevelyan et al., 1950). This reagent can detect approx. 25 µg of sulphated disaccharide but produces some diffusion of the spots. This report describes the use of $p$-anisidine ($p$-methoxyaniline) as a coloured detection probe for nano- and micro-gram quantities of chondroitin sulphate disaccharides after separation by paper electrophoresis.

Human articular-cartilage chondroitin sulphate and purified standards were incubated with chondroitin ABC lyase (Seikagaku Kogyo Co., Tokyo 103, Japan) in 0.1 M-acetate/Tris buffer, pH 7.90, at 37°C for 90 min (Saito et al., 1968). Some samples were subsequently treated with chondro-4-sulphatase to remove the sulphate from the 4-sulphated disaccharide (Suzuki et al., 1968). The electrophoretic separation of unsulphated and 4- and 6-sulphated disaccharides was on Whatman no. 1 chromatography paper (32 cm × 17 cm). Samples (5 µl) were applied to the buffer-saturated paper 6 cm from the cathode, permitting a migration distance of 18 cm to the anode. The electrophoresis buffer was 0.05 M-citrate, pH 4.00 (Hata & Nagai, 1972). Separation of the disaccharides was obtained by using 20 mA (200-300 V) for 4.5 h. The paper was then dried at 75°C for 1 h. The detection reagent was prepared by dissolving 1 g of $p$-anisidine/
HCl in 10 ml of methanol and diluting to 100 ml with butan-1-ol; just before use, 0.1 g of sodium dithionite was added. The dried electrophoresis sheets were sprayed with the reagent and developed at 120°C for 15 min.

With 4- and 6-sulphated disaccharides a strong colour-positive reaction occurred with p-anisidine at the highest amount examined (20 µg) and a detectable coloured product was observed at the lowest amount (156 ng). The unsulphated disaccharide produced a weaker reaction, but was detected at a concentration of 1 µg. The p-anisidine reagent provided more than 50 times the sensitivity of alkaline AgNO₃. The best of several alkaline AgNO₃ reagents examined was that described by Trevelyan et al. (1950). Cartilage extracts of glycosaminoglycans, enzymes and buffers did not cause interference with the disaccharide-anisidine reaction. In an examination of the monosaccharide components of glycosaminoglycans, the amino sugars, glucosamine and galactosamine did not react with p-anisidine. Glucose, galactose and uronic acids gave a positive (brown) reaction confirming previous reports that this reagent reacts with reducing monosaccharides (Merck, 1966). Under the assay conditions described monosaccharides were not produced from the chondroitin sulphate.

The electrophoresis conditions described produced migration distances of 9.5 cm for unsulphated disaccharide with the 4-sulphated disaccharide located between 12.5 and 14.5 cm and the 6-sulphated disaccharide between 13.0 and 15.0 cm. The desulphation of the 4-sulphated disaccharide by chondro-4-sulphatase readily permitted separation and identification of mixtures containing the two disaccharides.

The depolymerization of chondroitin sulphate from articular cartilage extracts with chondroitin ABC lyase produced p-anisidine-detectable sulphated disaccharides after 45 min at 37°C. The nature of the reaction between p-anisidine and the chondroitin sulphate disaccharides was not resolved. For the quantitative determination of microgram quantities of 4- and 6-sulphated disaccharides the periodate/thiobarbituric acid and borate-$\beta$-dimethylaminobenzaldehyde assays were used (Elliott & Gardner, 1977).

Evidence for the Presence of a Calcium-Ion-Binding Site within Fibrinogen Fragment D

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Recent reports have emphasized the importance of Ca²⁺ to native fibrinogen structure. The results of Marguerie (1977) and Marguerie et al. (1977) suggest the existence of three strongly bound Ca²⁺ ions per fibrinogen molecule. The position of these within the molecule is not established, but Marguerie (1977) has suggested that a binding site exists in the C-terminal portion of the (A)a-chain. Haverkate & Timan (1977) showed that in the presence of Ca²⁺ the digestion of fibrinogen by plasmin proceeded more slowly than in their absence, and, significantly, that only a single molecular-weight species of fragment D was produced. This is in contrast with the digestion in the absence of Ca²⁺, where three D fragments of mol.wts. 94000 (D₁), 88000 (D₂) and 83000...