The Application of a Lipophilic Dextran Derivative to Analysis of Plant Terpenoids

By R. A. B. KEATES and C. J. W. BROOKS. (*Department of Chemistry, University of Glasgow, Glasgow W.2*)

Gels of hydroxyalkoxypropyl-dextran ('modified LH-20'), made by reaction of terminal epoxy-alkanes with Sephadex LH-20 (Ellingboe, Nyström & Sjövall, 1968), swell in non-polar solvents such as benzene. Our observations indicate that the separating properties of the benzene-gel preparation involve gel filtration. The selective retention of aromatic compounds, observed for Sephadex G-25 and LH-20 (Janson, 1967), is absent: there is, however, a powerful hydrogen-bonding effect, the gel acting exclusively as the basic component. Hydroxyl groups markedly increase retention to a degree dependent on their number and steric environment. Carboxylic acids are severely retained. The incorporation of an alcohol in the benzene permits selective displacement of such samples, apparently by competitive hydrogen bonding. Propan-2-ol-benzene (1:3, v/v) gives negligible retention of monohydroxylic compounds. Columns (100 cm. $\times$ 0.9 cm. diam.) of 'modified LH-20' had theoretical plate heights 0.12–0.14 mm. The composition of eluates was determined by gas-liquid chromatography, which demonstrated that the compounds were eluted as symmetrical peaks. Examination of radioactive samples indicated the virtual absence of 'tailing'. We define standard elution volume (S.E.V.) as the elution volume for a column of bed volume 100 units; this allows the correlation of results from columns of various dimensions. Standard elution volumes are found to be reproducible, to s.e.m. $\pm$ 0.5% (internal standard: cholesteryl acetate, S.E.V. 56.3). Examples include isopetasyl acetate (a sesquiterpenoid ester), S.E.V. 59.1; lanosterol (a hindered alcohol), 82.3; cholesterol, 110.2; and isopetasol, 119.6.

Crude plant extracts have previously shown poor separations on unmodified Sephadex LH-20: for example, selective retention of highly conjugated compounds causes pigments to be eluted with material of lower molecular weight (Ruzicka, Thomson, Wheals & Wood, 1968). By using columns of 'modified LH-20' in benzene, these pigments are eluted largely in early fractions. Most terpenoids are eluted later by the same solvent. Highly polar compounds (glycosides, free acids etc.) remain until eluted by alcohol-benzene mixtures.

As shown by Sjövall, Nyström & Haahti (1968), the application of gel chromatography offers a number of advantages. Analytical scale (10 μ g.–

10 mg.) results may be reproduced preparatively (10 mg.–1 g.) with little loss in efficiency. The mild conditions are particularly suited to samples susceptible to decomposition, and it is unnecessary to prepare derivatives of highly polar or non-volatile compounds.

We are indebted to Dr J. Sjövall for encouraging our interest in modified dextrans.

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The Use of Cellulose Phosphate in the Extraction of Free Nucleotides from Plant Tissue

By A. J. KEYS and G. R. ROBERTS. (*Agricultural Research Council Unit of Plant Physiology, Imperial College of Science and Technology, London S.W.7*)

The quantitative extraction of certain nucleotides from plant tissues requires the use of a metal-complex-forming agent (Cole & Ross, 1966; Isherwood & Barrett, 1967; Keys, 1968). For this purpose 1,1,1-trifluoro-3-(2-thenoyl)acetone was used (Keys, 1968) because it and its metal complexes are soluble in methanol and may be washed away, leaving nucleotides in the tissue residue. Difficulties were encountered in this procedure and an insoluble polymer was sought to which metal-binding groups were attached. Enquiries lead to the successful use of the cellulose phosphate derivative, Whatman Column Chromedia P 11.

Freeze-dried samples of powdered tobacco leaf were treated with boiling ethanol and phenazine methosulphate (Keys, 1968). Cellulose phosphate (0.5 g.), previously washed and freed from fine particles, was added and the suspension transferred to a chromatography column that already contained 0.5 g. of cellulose phosphate mixed in acetone with 0.5 g. of Whatman CF1 cellulose powder. Extraction followed the method of Keys (1968) except that 1,1,1-trifluoro-3-(2-thenoyl)acetone and ammonium acetate were omitted from extracting solvents. Inclusion of ammonium acetate, pH 7.4, especially where this was sufficient to saturate the cellulose phosphate with ammonium ions, caused up to 90% of the UDP-glucose in samples to be lost. No UDP or UMP was formed to account for the loss of UDP-glucose. Added 14 C-labelled UDP-

glucose was not lost, suggesting that close association of the nucleotide with the tissue residue was involved. Replacing the ammonium acetate with acetate buffers of pH below 4 resulted in some retention of nucleotides by the tissue residue, presumably by ionic binding to protein. Similar but much smaller effects were produced in the absence of cellulose phosphate. We found that P11 cellulose phosphate washed and equilibrated with acetate buffer of pH 5.0 was a satisfactory alternative to 1,1,1-trifluoro-3-(2-thenoyl)acetone and gave good recoveries of the nucleotides, including UDP-glucose, without the risk of contamination that attended the use of the soluble metal-complex-forming agent.

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Identification and Determination of Hydroxylysine in Connective-Tissue Proteins

By R. D. SHENTALL, F. HAPPEY and C. H. PEARSON)
 (Textile Department, University of Bradford) and
 A. NAYLOR and R. L. TURNER (Bradford Royal Infirmary)

A significant content of hydroxylysine has been tentatively reported in a non-collagenous fraction obtained from complete human intervertebral discs (Steven, Jackson & Broady, 1968) and protein-polysaccharide complexes derived from intercostal cartilage (Steven, Broady & Jackson, 1969). Robert and his colleagues have isolated a number of connective-tissue non-collagenous structural glycoproteins, which appear to contain considerable amounts of hydroxylysine (Robert & Comte, 1968). If hydroxylysine is present in these non-collagenous proteins, which are difficult to remove from the collagen, unreliable results for the hydroxylysine content of the associated collagen may be obtained.

Steven *et al.* (1968) isolated collagen from complete human intervertebral discs by using crude bacterial α -amylase. Most of the collagen was probably derived from the annulus fibrosus, because we have found this method ineffective when applied to the nucleus pulposus of the disc. However, a short treatment with porcine trypsin of the water-insoluble complexes from the nucleus pulposus released a non-diffusible non-collagenous glycoprotein (NT). Further digestion of the insoluble residue followed by dispersion in acetic acid and precipitation with sodium chloride gave a collagen apparently of 80–90% purity. The annulus fibrosus treated under similar conditions gave collagen of

purity comparable with that of insoluble tendon collagen (Steven & Jackson, 1967). Both disc collagens were higher in hydroxylysine content than most mammalian collagens.

Amino acid analysis, on the 130 cm. column of a Technicon apparatus, of the glycoprotein NT, which has been reported elsewhere (Pearson, Happey, Shentall, Naylor & Turner, 1969), showed one major peak (Xn) in the position expected for *n*-hydroxylysine. The following evidence proves that peak Xn is not 5-hydroxylysine. The ratio E_{440}/E_{570} for the ninhydrin reaction product was about 0.2 compared with about 0.5 for hydroxylysine. Elution with a single citrate buffer (pH 4.43, I 0.40) separated substance Xn from *allo*-hydroxylysine and *n*-hydroxylysine. The high-voltage electrophoretic mobilities of hydroxylysine and substance Xn at pH 1.85 were also considerably different. Under the same conditions that hydroxylysine was destroyed by periodate, substance Xn remained unchanged.

The compound reported as hydroxylysine by Steven and his co-workers in non-collagenous proteins from the disc and cartilage may be identical with the unknown component found by us in several similar preparations. The findings of Robert and his colleagues also require confirmation.

The identification of substance Xn is under investigation.

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Incorporation of Amino Acids into the Protein of Mitochondria Isolated from Soya-Bean Hypocotyls

By R. BAXTER. ('Shell' Research Ltd., Division of Biochemistry, Woodstock Agricultural Research Centre, Sittingbourne, Kent)

Early reports of amino acid incorporation *in vitro* by mitochondria isolated from higher plants were published (Webster, 1954; Das, Chatterjee & Roy, 1964) before the necessity of avoiding heavy bacterial contamination was realized (see Roodyn, 1968).

In view of the limited amount of information about protein synthesis by isolated higher-plant mitochondria (Lado & Schwendemann, 1967; Baxter & Hanson, 1968; Ellis & MacDonald, 1968)