5. The relevance of these observations to the problem of the protein–mucopolysaccharide linkage is discussed and the hypothesis put forward that it is due to ion-pair formation between sulphuric ester groups and the amidine groups of arginine residues.

I wish to thank Dr A. S. McFarlane for his hospitality at the National Institute for Medical Research and Dr T.-C. Tsao for many invaluable suggestions. The preliminary ultracentrifuge work was done at the Massachusetts General Hospital, Boston, and I am very grateful to Dr Karl Schmid and Dr Jerome Gross for making this possible and for the use of the machine. I am also indebted to Dr C. L. Oakley for the gift of the collagenase preparation.

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The Isolation of *allo*Hydroxy-L-Proline from Sandal (*Santalum album* L.)

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During the course of investigations on the nitrogen metabolism of plants by the radial ('circular') paper-chromatographic technique, the presence of considerable quantities of free proline in leaves of several plant species and hydroxyproline in the leaves of sandal was observed (Giri, Gopalkrishnan, Radhakrishnan & Vaidyanathan, 1952). Examination of paper chromatograms of an ethanolic extract of the leaves of *Santalum album* showed that the band corresponding to hydroxyproline was quite prominent and an attempt was therefore made to isolate the substance in a pure state. In the present paper an account is given of its isolation and identification as *allo*hydroxy-L-proline.

The procedure of Town (1928) was not found suitable in this case. However, a partial purification was initially effected by absorption over a column of Zeo-Karb H.I., whereby some neutral constituents and a few of the amino acids were removed. By chromatography of the eluates on cellulose powder and copper carbonate–alumina columns, the purification of the substance was sufficient to induce crystallization. Failure to isolate the amino acid by Town's procedure is believed to be due to the presence of an accompanying substance which yields a picrate (m.p. 335°) but is otherwise as yet unidentified.

The purified amino acid gave correct analytical values and melted at 248° (decomp.). The melting point as well as the optical rotation are in good agreement with values previously reported for *allo*hydroxy-L-proline. A mixed melting point with an authentic sample was unchanged. Further, both the samples were indistinguishable in their behaviour on paper chromatograms and also crystallized in the same characteristic broom-like manner on a microscope slide. Additional evidence as to their identity was provided by X-ray powder photographs.

EXPERIMENTAL

Identification of free amino acids in leaves of Santalum album

Extraction. Fresh leaves were dried in an oven at 50° and powdered (200 mesh/in.). A sample (0·2 g.) of the powder was extracted for 1 hr. in the cold with 75% (v/v) aqueous ethanol (2 ml.). After centrifuging, the supernatant was shaken with $CHCl_3$ (3 vol.). The aqueous layer containing all the amino acids was separated by centrifuging and examined by paper chromatography. Clear separation of the bands could be achieved with 10–20 μl. of the aqueous extract.

Paper chromatography. Whatman no. 1 filter-paper disks (25 cm. diam.) were used and the chromatograms developed

with *n*-butanol–acetic acid–water solvent (4:1:5 by vol.), as described by Giri & Rao (1952*a*, *b*). The dried papers, on spraying with 0·1% (w/v) ninhydrin in 95% (v/v) aqueous acetone and heating at 60° (30 min.), showed bands (Fig. 1) corresponding to the following amino acids: leucine + isoleucine, phenylalanine, valine + methionine, tyrosine, proline, alanine, glutamic acid, hydroxyproline, arginine, histidine, lysine + ornithine and an unidentified peptide. The characteristic yellow bands of proline and hydroxyproline were prominent.

Confirmatory tests. Specific tests for hydroxyproline were carried out on a radial chromatogram of the aqueous extract by the 'multiple sector' technique. While with the isatin reagent (Acher, Fromageot & Jutisz, 1950) proline gave a dark-blue colour, the band corresponding to hydroxyproline gave a lighter duck-egg blue. With Folin's reagent (Giri & Nagabhusanam, 1952) the hydroxyproline gave an orange band. The combined reagents, alloxan–isatin (Harris & Pollock, 1953) and isatin–*p*-dimethylaminobenzaldehyde also gave a positive test. The latter reagent constitutes a highly sensitive and specific test for hydroxyproline (Jepson & Smith, 1953).

Estimation of hydroxyproline by the method of Neuman & Logan (1950) gave 1 mg./ml. of the aqueous extract employed for chromatography and this agreed well with the value (0·99 mg./ml.) obtained after separation of the amino acid by radial paper chromatography as follows. Chromatograms with 10 μl. of the extract applied at four points near the centre of a Whatman no. 1 paper disk (25 cm. diam.) were developed with the butanol–acetic acid–water mixture. After drying, the opposite segments were sprayed with ninhydrin to serve as guide strips for the remaining pair, from which the bands corresponding to hydroxyproline were cut out, eluted with water and estimated as before.

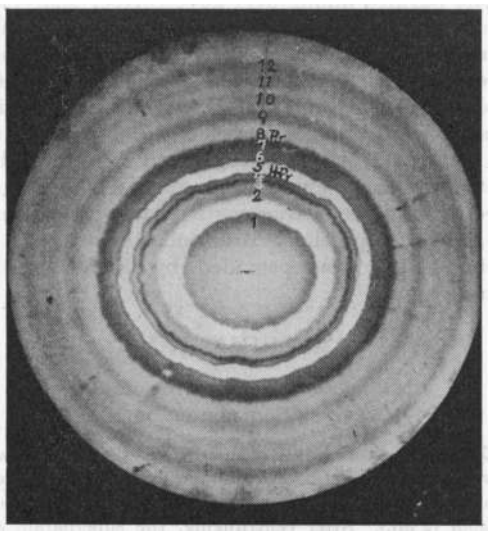

Fig. 1. Radial paper chromatogram of amino acids in ethanol extract of leaves of *Santalum album*, showing the position of *allo*hydroxy-L-proline. 1, Unidentified peptide; 2, lysine + ornithine; 3, histidine; 4, arginine; 5, hydroxyproline; 6, glutamic acid; 7, alanine; 8, proline; 9, tyrosine; 10, valine + methionine; 11, phenylalanine; 12, leucine + isoleucine. (For conditions see text.)

Isolation of allo*hydroxy-*L*-proline from leaves of* Santalum album

Material. Freshly collected sandal leaves free from twigs were cut, dried at 50° for 48 hr., and powdered in a mortar to 200 mesh/in.

Extraction. The powder (100 g.) was extracted for 2 hr. with hot 75% (v/v) aqueous ethanol (1 l.) and filtered. The residue was again extracted with the solvent (500 ml.). The combined extracts were evaporated to dryness *in vacuo*, taken up with water (20 ml.) and filtered to remove chlorophyll.

Purification over Zeo-Karb H.I. The dark-brown extract was placed at the top of a prepared column (2·5 × 50 cm.) of Zeo-Karb H.I. (Permutit Co. Ltd., London) and developed with water (200 ml.), when most of the amino acids (including hydroxyproline, the colouring matter and neutral constituents) were found in the eluate. On displacing the column by 10% (w/v) HCl, about 50% of the original solids was obtained as a partly crystalline mixture (containing only a small percentage of hydroxyproline) which was not further examined.

Fractionation of the amino acids by displacement chromatography. The aqueous eluate, which had a high concentration of hydroxyproline, was evaporated *in vacuo* to 20 ml. and fractionated on a second column of Zeo-Karb H.I. (2·5 × 50 cm.). On development with water, all the amino acids were absorbed leaving neutral materials and colouring matter in the eluate. The amino acids were displaced by 10% (w/v) aqueous HCl (300 ml.) and the effluent collected in 10 ml. fractions. The progress of fractionation of the amino acid was followed by radial paper chromatography. Hydroxyproline was present in the fractions 6–21, mixed with small quantities of other amino acids. The fractions 8–15, containing histidine and arginine also, were pooled and purified further by paper chromatography. The remaining fractions were used for the cellulose powder and copper carbonate–alumina columns.

Isolation by paper chromatography. A sample from the fractions 8–15, containing about 8–10 mg. of hydroxyproline was applied at the centre of a Whatman no. 1 (or, better, no. 3) disk (35 cm. diam.) and developed with the *n*-butanol–acetic acid–water solvent. Good resolution was attained by repeated drying and development with fresh solvent. As before, guide strips of 0·5 cm. width sprayed with ninhydrin were used to indicate the position of the amino acid. The bands were cut out from the chromatograms, packed in a small column and extracted with 75% (v/v) aqueous ethanol. Evaporation of the solvent *in vacuo* gave a residue containing most of the hydroxyproline with only traces of histidine and arginine. Although it was chromatographically homogeneous, the residue gave low values for hydroxyproline as estimated by the method of Neuman & Logan. However, on treatment with glacial acetic acid or aqueous ammonia (sp.gr. 0·880), a ninhydrin-negative material, not resolved chromatographically from hydroxyproline, was left undissolved. Its chemical nature is still unknown. The acetic acid extract was evaporated *in vacuo* to dryness, the residue dissolved in the minimum quantity of water, decolorized with charcoal and precipitated by addition of absolute ethanol. By a repetition of this process the amino acid was obtained in a pure state.

Fractionation on a column of copper carbonate–alumina. The hydroxyproline-rich fractions 6, 7 and 16–21 suitably

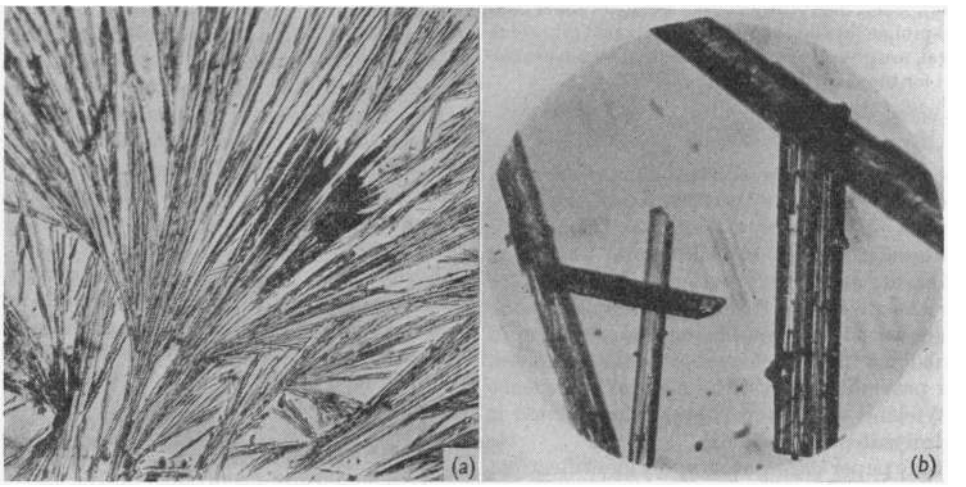

Fig. 2. Photomicrographs of crystals of natural *allo*hydroxy-L-proline: (*a*) as crystallized from aqueous ethanol on microscope slide; (*b*) as crystallized from *n*-propanol on slide (× 120).

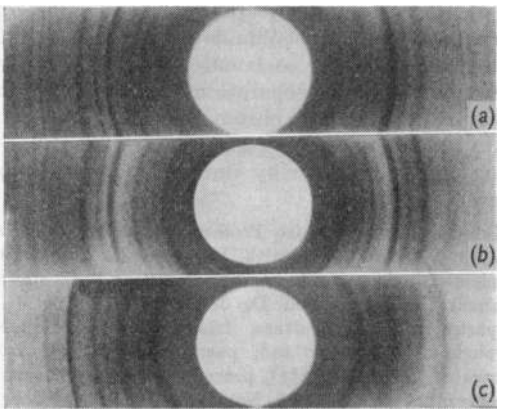

Fig. 3. X-ray powder photographs (paper positive) of hydroxyproline specimens (Cr radiation, V filter; camera diameter 5·7 cm.). (*a*) Authentic sample of *allo*-hydroxy-L-proline; (*b*) natural *allo*hydroxy-L-proline as obtained from *S. album*; (*c*) hydroxyproline.

diluted were passed through a column of basic copper carbonate–alumina (1:3) according to the method of Thompson, Pollard & Steward (1953). The basic copper carbonate used by us was a mixture of native malachite and azurite and the column was packed dry. The eluates were collected in 10 ml. fractions. Fractions 12–21, containing hydroxyproline, were freed from Cu^{2+} by passing H_2S. The residue obtained on evaporation *in vacuo* was purified as described before with glacial acetic acid.

Fractionation on a column of cellulose powder. Fractions 5–11 and 22–26 from the copper carbonate–alumina column containing mostly hydroxyproline were purified on a column of cellulose powder prepared according to Synge (1951). The solvent mixture, ethanol–acetic acid–water (90:2:8 by vol.), was employed both for preparing the column and for development. The effluent was collected in 10 ml. fractions. Hydroxyproline from fractions 10–24 was purified as before.

Crystallization. It was difficult to induce crystallization from aqueous ethanol unless the impurities were removed by treatment with glacial acetic acid or aqueous ammonia (sp.gr. 0·880). However, it was found that *allo*hydroxy-L-proline crystallizes best from aqueous *n*-propanol. By slow evaporation from this solvent at 50° it separated in thin, long, glistening needles. By all the methods employed above a total of approx. 100 mg. of the pure imino acid was obtained.

Identification of the natural amino acid as allo*hydroxy-L-proline.* The natural material was compared with synthetic *allo*hydroxy-L-proline (Robinson & Greenstein, 1952). The isolated amino acid was highly soluble in water and aqueous ethanol but practically insoluble in absolute ethanol and *n*-propanol. Both the natural and the synthetic samples of *allo*hydroxy-L-proline crystallized alike from aqueous *n*-propanol and Fig. 2 (*b*) shows a photomicrograph of the crystals. In Fig. 2 (*a*) can be seen the characteristic broom-like manner in which it crystallizes on a microscope slide from water or ethanol.

M.p. was 248° (uncorr., decomp.). When admixed with an authentic sample of *allo*hydroxy-L-proline (m.p. 246–247°) m.p. was 247°. Specific optical rotation (0·5 dm. microtube) was $[\alpha]_D$ −57° (water, $c = 0{\cdot}65$). Neuberger (1945) reported $[\alpha]_D$ −58°, m.p. 248°, and Wieland & Witkop (1940) $[\alpha]_D$ −57·4°, m.p. 241°.

Elementary analysis (Department of Organic Chemistry of this Institute) gave the following values. (Found: C, 45·6; H, 6·7; N, 10·5. Calc. for $C_5H_9O_3N$: C, 45·7; H, 6·9; N, 10·7%.)

Chromatographic behaviour. The isolated imino acid could not be resolved from *allo*hydroxy-L-proline on a chromatogram with the butanol–acetic acid–water solvent, nor with pyridine–water (4:1, v/v), phenol–water or ethanol–acetic acid–water (90:2:8 by vol.). Its behaviour with colour reagents was also identical with that of the authentic sample.

X-ray diffraction. The X-ray powder photographs of *allo*-hydroxy-L-proline (*a*) and the isolated compound (*b*), which are identical, are given in Fig. 3; that of hydroxy-L-proline (*c*) is given for the sake of reference.*

DISCUSSION

The experimental results obtained have furnished unequivocal evidence for the identity of the compound with *allo*hydroxy-L-proline. Using only paper-chromatographic identification, this was reported earlier as hydroxyproline (Giri *et al.* 1952). Thus for the first time free *allo*hydroxy-L-proline has been detected in plants, being now obtained in crystalline form from the leaves of *Santalum album*. The only previous report of its natural occurrence was by Wieland & Witkop (1940), who isolated it from hydrolysates of phalloidin.

Using only paper-chromatographic identification, hydroxyproline has further been reported to occur free in pollen (Auclair & Jamieson, 1948), in prunes (Joslyn & Stepka, 1949), in the haemolymph of *Drosophila melanogaster* (Auclair & Dubreuil, 1953), in the sporulation medium of *Bacillus globigii* (Davis & Williams, 1952) and in the blood and Malpighian tubes of the larvae of *Bombyx mori* infected with polyhedral disease (Drilhon, Busnel & Vago, 1951). Besides its well-known occurrence in collagens and gelatins, it has been reported in a combined state in alfalfa protein (Steward, Thompson & Millar, 1951), in sugar-beet protein (Sisakyan, Bezinger & Kuvaeva, 1951; Vavruch, 1952), in *Sarcina lutea* (Belozerskiĭ & Kireenkova, 1943), dentine protein (Losee, Neidig & Hess, 1951), proteins of the insect cuticle (Hackman, 1953) and horseradish peroxidase (Machly & Paleùs, 1950). Hydroxyprolines are not, however, found free or combined in most biological materials that have been examined by paper chromatography. Moreover, according to Steward & Thompson (1950), hydroxyproline has never been found free in plants. Chromatographically the isomers have not been differentiated. Since it is now known that besides these isomers, many other compounds behave somewhat similarly on paper chromatograms, previous claims regarding the presence of hydroxyproline in natural sources obviously require further substantiation, preferably by isolation.

*allo*Hydroxy-L-proline has been found to be present in sandal tree largely in the free state. It appears to be more concentrated in the flowers and the pericarp of the fruit than in the leaves. The proportion of hydroxyproline N to total N in samples of flowers and pericarp was found to be 31 and 45% respectively (Radhakrishnan, Gopalkrishnan & Giri, unpublished). Its presence in these materials may be related to its progressive movement from the leaves. In view of the occurrence of a high percentage of *allo*hydroxy-L-proline in the free state, *S. album* might be an economic source for its production on a large scale.

The role played by this amino acid in plant physiology is not known. A knowledge of the changes in the hydroxyproline content in normal and pathological (spiked) conditions of the tree will be of considerable physiological interest and work on this subject is in progress.

* Infrared spectra of *allo*hydroxy-L-proline and the natural preparation were studied by the kindness of Professor R. S. Krishnan, of the Department of Physics of this Institute, with a Perkin-Elmer single-beam automatic recording instrument, model 112, with rock-salt optics. The spectra were essentially identical in the region investigated (2–15μ.).

SUMMARY

1. The natural occurrence of free *allo*hydroxy-L-proline is reported for the first time. A detailed account is given of its isolation from the leaves of sandal (*Santalum album*).

2. The substance was isolated in crystalline form by radial ('circular') paper chromatography, chromatography on columns of cellulose powder and of basic copper carbonate–alumina, following a previous partial separation from other amino acids by ion-exchange chromatography.

3. The isolated amino acid has been identified as *allo*hydroxy-L-proline by numerous chemical and physical criteria.

Our thanks are due to Professor Jesse P. Greenstein (National Institute of Health, Bethesda) for a gift of the isomers of hydroxyproline and to Professor F. C. Steward (Cornell University) and Dr John F. Thompson (U.S. Department of Agriculture, Ithaca) for their interest throughout this work and, particularly, for supplying details of the $CuCO_3$–Al_2O_3 procedure before publication. We are indebted to Dr P. L. Narasimha Rao of this Department for helpful discussion. Our thanks are also due to Dr E. G. Ramachandran (Department of Metallurgy) for the X-ray photographs. One of us (A.N.R.) was a Government of India Senior Scholar during this work.

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Mode of Action of Vitamin D. The Effect of Vitamin D Deficiency in the Rat on Anaerobic Glycolysis and Pyruvate Oxidation by Epiphyseal Cartilage

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The role of vitamin D in calcification of bone still awaits elucidation; this is not to be wondered at since the mechanism of normal calcification is still far from clearly understood. The demonstration of alkaline phosphatase in bone and hypertrophic cartilage by Robison (1923) and Robison & Soames (1924) led to the suggestion that the enzyme promoted calcification by causing a local increase in concentration of inorganic phosphate. That this hypothesis did not explain fully the process of calcification was recognized by Robison, who postulated the presence of a second mechanism which was susceptible to the action of cyanide, fluoride and iodoacetate (Robison, MacLeod & Rosenheim, 1930; Robison & Rosenheim, 1934). Though Robison & Rosenheim (1934) did not make any suggestion about the nature of this second mechanism, in the light of the later knowledge of the action of the above-mentioned inhibitors in the intermediate metabolism of carbohydrates, it may be permissible to conclude that Robison's second mechanism must be very intimately connected with glycogen metabolism in hypertrophic cartilage.

Gutman & Gutman (1941) provided experimental evidence associating glycolysis with calcification occurring in calcifying cartilage. They demonstrated the presence of phosphorylase in significant concentration in the epiphyses of growing rats and rabbits and suggested that this enzyme could initiate conversion of the glycogen of hypertrophic cartilage cells into phosphoric ester substrates for cartilage phosphatase. Later Gutman, Warrick & Gutman (1942) observed that the addition of 0·0005 M phloretin, which is a specific inhibitor for phosphorylase, completely blocked the calcification of rachitic cartilage in solutions containing calcium and inorganic phosphorus in concentrations of 8 and 5 mg. respectively/100 ml. of the medium. The addition of glucose 1-phosphate (G 1-P) restored the capacity of the cartilage to calcify even in the presence of phloretin. The authors concluded, therefore, that phosphorylase was necessary in the calcification mechanism. Further work with the use of inhibitors like cyanide, iodoacetate and fluoride suggested that the calcification of cartilage involved phosphorylative glycogenolysis at least to the point of phosphopyruvate formation.

Additional evidence in support of the important role of glycogenolysis in initiating calcification was forthcoming from the work of Albaum, Hirschfeld & Sobel (1952*a*) who observed that adenosine triphosphate (ATP), which is essential in these enzymic processes, was present in epiphyseal cartilage of rat tibiae at the same level as was normally found in liver and kidney (9·2–17·2 μg./rat, i.e. two tibiae). Albaum, Hirschfeld & Sobel (1952*b*) further demonstrated the presence of most of the glycogenolytic enzymes in epiphyseal cartilage and several of the phosphorylated intermediates by a chromatographic technique.

Although the role of glycogenolysis in calcification had thus been clearly demonstrated, current opinion appears to view this process only from the standpoint of its being able to provide phosphoric esters, which under the influence of alkaline phosphatase would provide the requisite concentration of inorganic phosphorus for the deposition of the bone salt. This concept, which lays emphasis on the