66. *Studies on Fructosans. Part IV.* A Fructosan from* Dactylis glomerata.

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A fructosan from mid-season leafy cocksfoot grass (*Dactylis glomerata*) gave on hydrolysis D-fructose (97%) and D-glucose (3%). Methylation and hydrolysis yielded 1 : 3 : 4 : 6-tetramethyl D-fructose (4%), 2 : 3 : 4 : 6-tetramethyl D-glucose (1·8%), 1 : 3 : 4-trimethyl D-fructose (93·3%), and a dimethyl D-fructose (0·7%). The greater part of the material possesses therefore a molecular structure comprising a straight chain of *ca.* 25 2 : 6-linked fructofuranose residues terminated by a glucopyranose residue linked as in sucrose.

Fructosans of the levan type have been isolated from various plants such as rough-stalked meadow grass (*Poa trivialis*) (Challinor, Haworth, and Hirst, *J.*, 1934, 1560), barley leaves (Haworth, Hirst, and Lyne, *Biochem. J.*, 1937, **31**, 786), the roots of timothy-grass (*Phleum pratense*) (Schlubach and Sinh, *Annalen*, 1940, **544**, 101), and perennial rye-grass (*Lolium perenne*) (Laidlaw and Reid *). The present investigation has shown the fructosan from mid-season leafy cocksfoot grass (*Dactylis glomerata*) to possess a similar structure. Previous observations by Bell and Palmer (*Biochem. J.*, 1949, **45**, xiv) suggested a repeating unit of 14 residues for this fructosan, although physical measurements of sedimentation and diffusion constants suggested a chain length of 32—33. Palmer (*Biochem. J.*, 1951, **48**, 389), by estimation of the glucose produced on hydrolysis and on the basis of one glucose residue per chain, put forward values of 29 and 37 for the chain lengths of different samples of leafy-cocksfoot levan.

The cocksfoot grass used was oven-dried milled material prepared from grass cut in May, 1949, at the Jealott's Hill Agricultural Research Station. After preliminary extraction with ether and 80% aqueous methanol, the fructosan was extracted from the grass with water. Hydrolysis of the purified polysaccharide and analysis of the product by paper

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chromatography (Hirst and Jones, *J.*, 1949, 1659; Duff and Eastwood, *Nature*, 1950, **165**, 848) showed the presence of fructose (97%) and glucose (3%).

The fructosan was methylated under nitrogen with sodium hydroxide and methyl sulphate, and the product (OMe, 44·6%) was fractionated by dissolution in chloroform–light petroleum. Fraction 3 (OMe, 45·2%) was hydrolysed and the mixture of sugars separated on a cellulose column (Hough, Jones, and Wadman, *J.*, 1949, 2511). Two fractions (A and B) were obtained, corresponding to those previously observed on the paper chromatogram to be tetramethyl fructofuranose (containing some tetramethyl aldose) and a trimethyl fructose. Further elution yielded a small amount of a dimethyl fructose, which travelled at the same rate on the chromatogram as 3 : 4-dimethyl D-fructose. The tetramethyl fructofuranose was shown colorimetrically (cf. Arni and Percival, *J.*, 1951, 1822) to be present to the extent of *ca.* 4% in the hydrolysate. Its identity was confirmed by conversion into the crystalline tetramethyl D-fructofuronamide (Avery, Haworth, and Hirst, *J.*, 1927, 2313). Hypoiodite oxidation showed the presence of *ca.* 15% of tetramethyl aldose in A, and extraction of the remainder of the syrup with light petroleum gave a residual syrup from which some 2 : 3 : 4 : 6-tetramethyl D-glucose crystallised on seeding. Fraction B (93·3%) crystallised completely and proved to be 1 : 3 : 4-trimethyl D-fructose. Hypoiodite oxidation indicated at most only traces of aldose derivatives in this fraction.

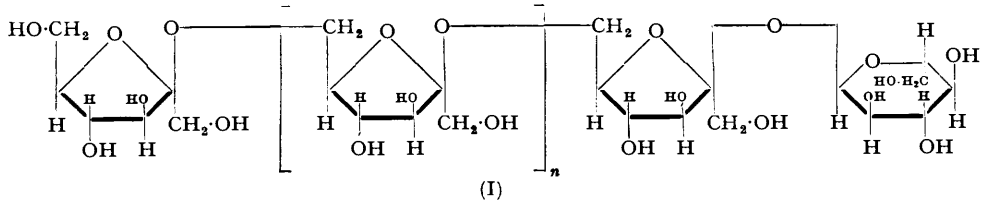
In order to attempt a quantitative separation of tetramethyl glucose a further quantity of methylated fructosan was hydrolysed, and the resulting sugars converted into the corresponding glycosides. Extraction in a liquid-extractor with light petroleum (Brown and Jones, *J.*, 1947, 1344) gave only partial separation of tetramethyl methylfructoside from tetramethyl methylglucoside. Each fraction was hydrolysed and the free sugars were separated on cellulose columns. Complete separation of tetramethyl glucose from tetramethyl fructose was eventually achieved by partition chromatography. The tetramethyl aldose was identified as 2 : 3 : 4 : 6-tetramethyl D-glucopyranose, and the total quantities present in the various fractions were estimated by hypoiodite oxidation. The presence of a trace of trimethyl aldose in the trimethyl fructose fraction was shown both by hypoiodite oxidation and by paper chromatography, but the quantity (*ca.* 1 mg.) was without structural significance. A small quantity of a dimethyl fructose was also obtained from the column but this substance was not present in sufficient amount for complete identification.

From these experiments the proportions of the various methylated sugars produced on the hydrolysis of the methylated fructosan are tetramethyl fructofuranose 4·0%, tetramethyl glucopyranose 1·8%, trimethyl fructofuranose 93·3%, and dimethyl fructose 0·7%. The proportion of tetramethyl fructose indicates a chain of *ca.* 25 fructofuranose units in the fructosan. The quantity of dimethyl fructose is too small to be of structual significance and probably arises from undermethylation of the polysaccharide and/or demethylation during the hydrolysis. The quantity of tetramethyl glucose isolated, although not accounting for all the glucose present, does not permit of one residue per chain but suggests that a large proportion of fructosan molecules are terminated by a non-reducing glucose residue probably present in a sucrose-type linkage. It is possible that some degradation took place during the isolation and methylation of the polysaccharide with scission of some terminal glucose units. The absence of all but traces of other methylated glucoses eliminates the possibility of a contaminating glucosan's being present with the fructosan. Measurement of the molecular weight of the methylated fructosan by Barger's method (cf. Caesar, Gruenhut, and Cushing, *J. Amer. Chem. Soc.*, 1947, **69**, 617) gave a value corresponding to 17—25 $C_9H_{16}O_5$ units.

Oxidation of the polysaccharide consumed *ca.* 1·02 moles of sodium metaperiodate per $C_6H_{10}O_5$ residue, in agreement with the postulate of a molecule composed of fructofuranose residues linked through the 2 : 6-positions. Oxidation with potassium periodate (Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27) yielded 1 mole of formic acid per 19—20 $C_6H_{10}O_5$ residues.

Heating the fructosan in water at 100° caused autohydrolysis. The fall in pH and the change in specific rotation were followed and samples were examined on the chromatograms as the reaction proceeded, spots being obtained corresponding to sucrose, glucose, fructose,

and three unidentified acids. The " autohydrolysis " is at least in part a breakdown of the fructosan by acid hydrolysis.



(I)

These experiments essentially confirm the structure deduced from methylation data and show that this fructosan possesses a structure (I) very similar to that proposed for the fructosan from perennial rye-grass (Laidlaw and Reid, *loc. cit.*). The production of formic acid on periodate oxidation is slightly greater (1 mole per 19—20 $C_6H_{10}O_5$ residues) than would be expected if all the chains are terminated by a glucopyranose residue (1 mole per 25 residues). If, as has been suggested, scission of some of the terminal residues has occurred then the yield of formic acid from a reducing fructofuranose residue would explain the observed increase.

The structure of the fructosan as a chain of fructofuranose units linked through the 2 : 6-positions and terminated by a glucopyranose unit linked as in sucrose is in full agreement with the theories put forward by Dedonder (*Compt. rend.*, 1950, **230**, 549, 997; 1951, **231**, 790; **232**, 1134, 1442) and by Bacon and Edelman (*Biochem. J.*, 1951, **48**, 114; **49**, 446, 529) that fructosans are built up in the plant from sucrose by enzymic trans-fructosidation.

Experimental

Preparation and Properties of the Polysaccharide.—Portions (100 g.) of mid-season cocksfoot grass (oven dried; H_2O, 9·8%; cut, May 26th, 1949) were extracted with ether (1 l.) for 5 hours. The residue was filtered and dried, then extracted with 80% aqueous methanol for a further 5 hours, after which the grass was filtered off and extracted for 3 hours with fresh 80% aqueous methanol (1 l.). The residue was removed, dried in air, and shaken with distilled water (1·3 l.) for 12 hours at room temperature. The grass was removed by filtration and washed with water, and the extract and washings (*ca.* 300 c.c.) were combined. The solution was heated to 93°, cadmium sulphate solution (40 c.c.; 10%) and sodium hydroxide solution (20 c.c.; 0·5N) were added, and the whole was kept at 95° for 3 minutes. After cooling, the cadmium hydroxide–protein complex was removed by filtration through " Filter Cel " (cf. Doak, *N.Z. J. Sci. Tech.*, 1939, **21**, 90B). The clear filtrate was de-ionised on columns of Amberlite resins (I.R.-100 and I.R.-4B), and the last traces of protein were removed by chloroform (0·25 vol.)–butanol (0·1 vol.) (Sevag, Lackmann, and Smollens, *J. Biol. Chem.*, 1938, **124**, 425). Evaporation of the neutral solution to 100 c.c. gave a light-amber coloured solution, which was poured into methanol. The colourless fructosan precipitated was removed at the centrifuge, washed twice with acetone, and dried in a vacuum-desiccator (P_2O_5 and paraffin wax). The polysaccharide was thus obtained as a fine colourless powder. By repetitions 30 g. of fructosan were prepared and combined. The average yield was *ca.* 3% of the dry weight of the grass. The fructosan had $[\alpha]_D^{15}$ −40·4° (*c*, 1·7 in H_2O) (Found: ash, 0·37; as sulphate, 0·45; N, 0·05%), and slowly reduced boiling Fehling's solution.

The fructosan (0·149 g.) was heated in 0·1N-oxalic acid (10 c.c.) at 70°, the following changes being observed: $[\alpha]_D^{15}$ −40·3° (0 min.); −46·3° (10 min.); −67·1° (20 min.); −78·5° (30 min.); −83·9° (40 min., const.). Paper chromatograms run in ethyl acetate–water–acetic acid (4 : 4 : 1 and 6 : 6 : 1) showed the hydrolysate to contain only fructose and a little glucose.

Hydrolysis of the polysaccharide until the rotation became constant ($[\alpha]_D^{16}$ −85° in 0·1N-oxalic acid), followed by neutralisation, filtration, de-ionisation, and concentration, gave a reducing solution which was shown chromatographically to consist of fructose *ca.* 97% and glucose *ca.* 3%. The fructose was estimated by oxidation with sodium metaperiodate as described by Hirst and Jones (*loc. cit.*), and the glucose by the Nelson colorimetric method (Nelson, *J. Biol. Chem.*, 1944, **153**, 375; Duff and Eastwood, *loc. cit.*). A synthetic glucose–fructose mixture containing 2·9% of glucose gave a recovery of 3·1% of glucose by this method.

Acetylation.—A specimen of polysaccharide (1·5 g.) was dissolved in dry pyridine (20 c.c.)

according to the method of Pacsu and Mullen (*J. Amer. Chem. Soc.*, 1941, **63**, 1487). Acetic anhydride (20 c.c.) was added with stirring during 7 hours and the solution left for 2 days. The acetylated fructosan was precipitated with water (1 l.) and washed with water, and the product dried in a vacuum-desiccator ($CaCl_2$), giving a white powder. After reprecipitation from chloroform with light petroleum (b. p. 60—80°), a fine white powder was obtained {1·8 g.; $[\alpha]_D^{16}$ +22° (*c*, 1·1 in $CHCl_3$); $\gamma_{sp.}^{20}/c' = 1·09$ where *c'* is the concn. in moles of the unit $C_{12}H_{16}O_8$ per l.} (Found : Ac, 43·1%).

To a solution of this acetyl derivative (0·5 g.) in chloroform (2·5 c.c.), cooled in a freezing mixture, a solution of sodium (0·25 g.) in absolute methanol (1 c.c.) was added (cf. Zemplen and Pacsu, *Ber.*, 1929, **62**, 1613). The mixture was shaken for 5 hours and ice-water (1 c.c.) added, followed by acetic acid (0·5 c.c.; 10%). Water (4 c.c.) was then added and the solution left to separate overnight. The regenerated fructosan was precipitated from the aqueous layer, with methanol. The product (0·22 g.) showed $[\alpha]_D^{15}$ −40·2° (*c*, 1·1 in H_2O) and mild acid hydrolysis followed by chromatographic examination showed the presence of fructose and glucose only.

Periodate Oxidation.—The fructosan (0·2864 g.) was dissolved in water (35 c.c.) and sodium metaperiodate (15 c.c.; 0·3M) added. The periodate uptake, determined by the arsenite method, was constant after 1 day at a value of 1·02 moles of periodate per $C_6H_{10}O_5$ residue.

Oxidation with potassium metaperiodate gave the following results (expressed as the number of $C_6H_{10}O_5$ residues per mole of formic acid liberated) : 22·8 (73 hr.); 21·4 (100 hr.); 20·3 (126 hr.); 19·5 (171 hr., const.).

The phenylhydrazine hydrochloride–potassium ferricyanide colour reaction with the solution of periodate-oxidised polysaccharide was negative, indicating the absence of formaldehyde.

Methylation.—The fructosan (10 g.) was methylated in the usual way with methyl sulphate and sodium hydroxide solution under nitrogen at room temperature. After the first methylation, the partly methylated material was separated, dispersed in acetone, and further methylated with methyl sulphate and sodium hydroxide solution. The methylation in acetone was repeated twice, giving a product (9 g.) showing OMe 44·6%.

Fractionation of the methylated fructosan was effected by refluxing chloroform–light petroleum (b. p. 40—60°) mixtures of varying composition. The following results were obtained.

Fraction	% $CHCl_3$ in solvent	$[\alpha]_D^{15}$ (*c*, 1·1 in $CHCl_3$)	OMe, %	Wt. (g.)
1	27	−54°	45·3	0·4
2	30	−54	45·3	0·6
3	32·5	−56	45·2	4·5
4	35	−56	45·5	1·6

Fractions 1, 2, and 4, together with some unfractionated material, were combined and methylated with silver oxide and methyl iodide to give a product (Fr. X) which showed OMe, 45·4%, $[\alpha]_D^{16}$ −54·4° (*c*, 1·2 in chloroform).

Hydrolysis of Methylated Fructosan.—The methylated fructosan (Fr. 3 above, 3·209 g.) was heated with methanol (100 c.c.) and water (34 c.c.) containing oxalic acid (1·35 g.) at 80° for 24 hours, and a little insoluble material (0·187 g.) was filtered off. Water (300 c.c.) was then added gradually and the water–methanol mixture removed at 40° under diminished pressure. The solution was reduced to its original volume and heated at 80° for 5 hours, neutralised with calcium carbonate, and filtered, and the filtrate evaporated to small volume at 35° under diminished pressure. The mobile syrup was extracted several times with boiling chloroform, and the extracts were combined, dried (Na_2SO_4), and taken to small volume. Examination of the resultant pale yellow syrup on the paper chromatogram, with butanol–ethanol–water solvent and developing sprays of aniline oxalate and urea oxalate, showed the presence of four sugars, corresponding to tetramethyl fructofuranose, tetramethyl glucose, a trimethyl fructose, and a dimethyl fructose, the latter being in very low concentration (R_G : 1·00; 1·00; 0·84; 0·62).

The insoluble material from the above hydrolysis, on further hydrolysis, gave paper chromatograms identical with those described above.

Separation of Methylated Sugars.—The sugars arising from the hydrolysis of fraction 3 were separated on a cellulose column (70 × 3 cm.) with light petroleum (b. p. 100—120°)–butanol (7 : 3), saturated with water, as eluant (Hough, Jones, and Wadman, *loc. cit.*), to give Fractions A and B.

Fraction A contained tetramethyl fructofuranose and tetramethyl glucose only. The tetramethyl fructofuranose (110 mg.) was estimated colorimetrically (Arni and Percival, *loc. cit.*), and hypoiodite oxidation showed that tetramethyl glucose was present to the extent of 17 mg.

Fraction A (OMe, 49·4%; n_D^{20} 1·4500) showed $[\alpha]_D^{17}$ +39° (*c*, 0·99 in H_2O). *Ca.* 40 mg. of this

fraction were oxidised with nitric acid and converted into the crystalline tetramethyl D-fructofuronamide, according to the method of Avery, Haworth, and Hirst (*loc. cit.*). After three recrystallisations from ether–light petroleum needles were obtained, of m. p. 99—101° (not depressed on admixture with an authentic specimen) (Found: C, 48·7; H, 7·7; OMe, 46·8. Calc. for $C_{10}H_{19}O_6N$: C, 48·2; H, 7·6; OMe, 49·8%).

The remainder of the syrup from fraction A was extracted with light petroleum (b. p. 35°), and both the extract and syrupy residue were taken to dryness. A speck of authentic crystalline tetramethyl glucose was added to the syrups, which were kept in a vacuum-desiccator over phosphoric oxide and paraffin wax, in a refrigerator for 3 weeks. The " extract " remained a syrup, but the " residue " crystallised partially. The crystals, separated on porous tiles, had m. p. 82°, not depressed on admixture with an authentic specimen of tetramethyl glucose.

Fraction B (2·211 g.) crystallised completely when kept at 0°. Chromatographic examination showed the absence of aldose. The sugar showed m. p. 73—75° after two recrystallisations from carbon tetrachloride–light petroleum, not depressed on admixture with authentic 1 : 3 : 4-trimethyl D-fructofuranose. It had $[\alpha]_D^{16}$ (*c*, 1·4 in H_2O) −27·4° (4 min.), −47·3° (20 min.), −56·6° (60 min.); −58·8° (5 hr.), −60·6° (20 hr.), −61·1° (68 hr., const.) (Found: C, 48·9; H, 8·4; OMe, 40·6. Calc. for $C_9H_{18}O_6$: C, 48·6; H, 8·2; OMe, 41·9%). Examination by hypoiodite oxidation showed the absence of aldoses. Periodic acid oxidation (Reeves, *J. Amer. Chem. Soc.*, 1941, **63**, 1476) gave 0·8 mole of formaldehyde per mole of trimethyl sugar, estimated as the formaldehyde–dimedon compound (m. p. and mixed m. p. 186—187°).

A very small third fraction (*ca.* 7 mg.) was also obtained from the column, chromatographic examination of which showed the presence of only one sugar travelling at the same rate as 3 : 4-dimethyl fructose.

Hydrolysis of Methylated Fructosan X.—The methylated fructosan (2·512 g.), prepared as detailed above, was hydrolysed with methanolic oxalic acid as before; the solution was neutralised with calcium carbonate and filtered. The filtrate was concentrated, methanol added, and the water–methanol mixture distilled off with constant addition of methanol. The volume was taken to *ca.* 5 c.c., methanolic hydrogen chloride (50 c.c.; 0·3%) added, and the whole shaken, set aside at room temperature for 3 hours, neutralised, filtered, and freed from methanol as before. The aqueous solution (50 c.c.) was extracted, in the presence of a little barium carbonate, with purified light petroleum (b. p. 38—40°) in a liquid-extractor (Brown and Jones, *loc. cit.*) for periods of 10, 12, and 16 hours. The three extracts were evaporated practically to dryness and hydrolysed separately with sulphuric acid (15 c.c.; N/5) for 6 hours at 80°. The first contained only ketose, but the second and third contained also small quantities of tetramethyl aldose. The hydrolysed extracts were combined, to give fraction *a*. The aqueous solution in the extractor was filtered, taken to small volume, and hydrolysed with sulphuric acid (250 c.c.; N/5) for 6 hours at 80°, to give fraction *b*. Chromatographic examination of the products showed a high proportion of trimethyl fructose, with small amounts of tetramethyl fructose and tetramethyl glucose.

Separation of Fraction a.—The sugars in this fraction were separated into two fractions, a_1 and a_2, on a cellulose column (60 × 1·8 cm.) with light petroleum–butanol as before. Fraction a_1 contained tetramethyl fructofuranose (60 mg.) and tetramethyl glucose (13·4 mg.) estimated as described above. Fraction a_2 (0·228 g.) crystallised completely; hypoiodite oxidation indicated the presence of a small quantity of aldose, but aldoses could not be detected on a paper chromatogram, run in benzene–ethanol–water (167 : 45 : 15) (Andrews, Hough, and Jones, *J.*, 1952, 2746), it having been shown that 1 : 3 : 4-trimethyl fructose and 2 : 3 : 4-, 2 : 4 : 6-, and 2 : 3 : 6-trimethyl glucoses were separated under those conditions. No dimethyl sugars were obtained from fraction *a*.

Separation of Fraction b.—Separation of this fraction into its components was attempted by elution through a cellulose column (66 × 2·2 cm.) with benzene–ethanol–water, this solvent having been found capable of separating tetramethyl glucose from tetramethyl fructose on the paper chromatogram. Three main fractions, b_1, b_2, and b_3, were collected. Fraction b_1 contained tetramethyl glucose, tetramethyl fructose, and a little trimethyl fructose, separated on 4 sheets of 3 M.M. filter-paper by the above-mentioned solvent. The tetramethyl fructose, estimated colorimetrically, amounted to 30·6 mg., whilst alkaline hypoiodite showed the presence of 29·0 mg. of tetramethyl glucose. The trimethyl fructose amounted to 22 mg. Fraction b_2 (1·862 g.) was twice crystallised from carbon tetrachloride–light petroleum and the supernatant liquors from each crystallisation combined and examined on the paper chromatogram; a small quantity of trimethyl aldose was observed (*ca.* 1 mg. as determined by alkaline hypoiodite oxidation). Fraction b_3 was a mixture of trimethyl fructose and dimethyl fructose, with a trace of trimethyl glucose; these were separated on a cellulose column (56 × 1·6 cm.), elution with

light petroleum–butanol (7 : 3) saturated with water as eluant and then with light petroleum–butanol (1 : 1) yielding trimethyl fructose, 36·0 mg., and dimethyl fructose, 16·4 mg.

Identification of Tetramethyl Glucose.—The aldose material from fraction b_1 after two crystallisations from ether–light petroleum (b. p. 40—60°) had m. p. 85°, not depressed on admixture with an authentic specimen of 2 : 3 : 4 : 6-tetramethyl D-glucose, $[\alpha]_D^{15}$ +95° ⟶ +84° (*c*, 0·5 in H_2O) (Found : OMe, 51·4%).

Examination of Dimethyl Fructose.—This had R_G 0·61 [butanol–ethanol–water (40 : 10 : 50)], OMe, 27·2%, and n_D^{20} 1·4784.

Molecular Weight by Barger's Method.—Barger's capillary technique (*loc. cit.*) was employed to determine the molecular weights of the acetylated and methylated derivatives of the fructosan. Droplets of solutions of known concentration were compared with solutions of sucrose octa-acetate (1—6 × 10^{-3}M). No satisfactory results could be obtained with 4%, 2%, and 1% solutions of the acetate, but a 1% solution of the methylated fructosan (fraction 3) gave reproducible values between 3440 and 5160, the equilibrium point being found to be between concentrations of sucrose octa-acetate of 2 × 10^{-3} and 3 × 10^{-3}M. An attempt to increase the accuracy of the method by employing a 2% solution of the methylated fructosan was unsuccessful.

Autohydrolysis of the Fructosan.—The fructosan (0·184 g.) in distilled water (25 c.c.) showed the changes at 100° : $[\alpha]_D^{15}$ −40·1° (initial value), −37·5° (4·5 hr.), −36·7° (9 hr.), −34·3° (14 hr.), −42·4° (19 hr.), −48·9° (22 hr.), −84·4° (29 hr.), −86·2° (35 hr.). After 22 hours a portion (15 c.c.) of the solution was chromatographically examined. It contained fructose, glucose, and three oligosaccharides, one of which travelled at the same rate as sucrose. Elution of this sugar from the paper, followed by hydrolysis, gave a solution containing two sugars, which travelled on the paper chromatogram at the same rate as glucose and fructose respectively.

After 30 hours, the pH of the solution was 3·4 (initial value 6·1) owing to the formation of three acids, observed chromatographically on running samples of the solution in butanol saturated with 1·5N-ammonia (Reich and Lederer, *Biochem. J.*, 1951, **50**, 60) and developing with methyl-red–methylene-blue (Conway and Byrne, *Biochem. J.*, 1933, **27**, 419).

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