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AN ENZYME–P.M.R.-SPECTROSCOPIC DETERMINATION OF THE ENANTIOMERS OF GALACTOSE*

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ABSTRACT

The action of D-galactose oxidase on D-galactose in the presence of oxygen afforded *meso-galacto*-hexodialdose quantitatively, which allowed p.m.r.-spectroscopic determination of the D enantiomer in a DL mixture. In the spectrum of the products obtained from the enzymic treatment of a mixture of D- and L-galactose, the magnitude of the aldehydrol signals derived solely from the oxidised D enantiomer, relative to those of the anomeric signals, provided the fractional content of the D enantiomer. This simple, accurate, and convenient procedure was applied to the hydrolysate of a seaweed galactan, which was also analyzed, for comparative purposes, by the fermentation technique employing D-galactose-adapted *Saccharomyces cerevisiae*.

INTRODUCTION

In the mammalian and plant kingdom, the D enantiomers of ribose, xylose, glucose, and mannose, and the L enantiomers of arabinose, fucose, and rhamnose are the most frequently encountered forms[1]. Galactose occurs much more frequently in both enantiomeric forms, particularly when associated with red algae[2], urchins[3], snails[4], and some higher plants[5]. The standard analytical procedures cannot differentiate between enantiomers, and fermentation techniques with selected strains of yeast have been used[6]. The complete fermentation of D-galactose by yeast[7] can only be achieved after first preconditioning most strains of *Saccharomyces cerevisiae* by culturing on a D-galactose-containing medium[8].

Other enzymes[9,10] have been employed to assess the content of D-galactose[11,12] and corresponding derivatives[13]. The dehydrogenase from *Pseudomonas fluorescens*, when admixed with NAD, oxidises D-galactose to the aldonic acid or 1,5-lactone, and has been employed to determine the content of L-galactose in the polysaccharides from flax seed, corn cobs, corn root, and the cell walls of cultured *Acer pseudoplatanus*[12]. The galactose oxidase isolated from the mold *Polyporus circinatus* or

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

Dactylium dendroides catalyses the oxidation of the primary carbinol group of D-galactose to the corresponding aldehyde[14], by stereospecific removal[15] of *pro-S* H-6. D-Galactopyranosyl units of various oligosaccharides and polysaccharides[16,17] have been oxidised by this D-galactose:oxygen oxidoreductase, and the reaction has been employed in the synthesis of 6-[^{3}H]-labelled D-galactopyranose residues[18,19].

The observations that the p.m.r. spectrum of the oxidation product from this enzyme reaction exhibited clearly defined signals associated with the oxidised carbinol function formed the basis of a simple, reliable, and convenient procedure for the quantitative determination of enantiomer composition of mixtures of D- and L-galactose. We now report on this method and its application to a hydrolysate of the sulphated galactan from the marine alga *Rhodymenia pertusa*[20,21].

RESULTS AND DISCUSSION

D-Galactose oxidase from *Dactylium dendroides*, as an oxygen oxidoreductase, differs from the corresponding D-glucose oxidase[22] in effecting an attack on the primary hydroxyl group rather than at the anomeric centre. The overall catalysed reaction is

$$\text{D-galactose} + O_2 \xrightarrow{\text{enzyme}} \textit{meso-galacto}\text{-hexodialdose} + H_2O_2 \downarrow \text{catalase}$$

However, it has been reported that the reaction is incomplete[19] and gives inconsistent quantitative results[10]. The experimental conditions described herein optimised the oxygen environment and involved an adequate concentration of hydrogen peroxide oxidoreductase so that the reaction was complete within 20 h.

The p.m.r. spectrum of a solution in deuterium oxide of the reaction products from the enzymic dehydrogenation of a mixture of 49.5% of D- and 50.5% of L-galactose is presented in Fig. 1. The two doublets at τ 4.43 and 4.46 originate from H-6 of the β and α anomers, respectively, of the *meso-galacto*-hexodialdose moiety. The $J_{5,6}$ value (7.1 Hz) of these doublets indicated that the aldehyde group existed as the hydrated (aldehydrol) form, and that H-6 and H-5 were antiparallel in the favoured rotamer. L-Galactose was not affected by galactose oxidase.

The dependence of the chemical shift of the aldehydrol proton on the configuration at the anomeric centre is illustrated by the spectra of the enzyme-treated methyl D-galactopyranosides, in which the signal for H-6 of the β anomer at τ 4.39 ($J_{5,6}$ 7.2 Hz) resonated to lower field than the corresponding signal for the α anomer (τ 4.42, $J_{5,6}$ 7.2 Hz). A similar, but smaller, displacement was observed in the spectra of the anomeric forms of methyl 2,3-di-*O*-methyl-D-*galacto*-hexodialdo-1,5-pyranoside, in which the signal for H-6 resonated at τ 4.40 for the β anomer and 4.41 for the α anomer.

Proof that the aldehyde at C-6 existed in the hydrated form in deuterium oxide followed from the p.m.r. spectrum of methyl 2,3-di-*O*-methyl-α-D-*galacto*-hexodialdo-1,5-pyranoside in chloroform-*d*, which contained a signal for a non-hydrated aldehydic proton as a singlet at τ 0.37. The zero value of $J_{5,6}$ indicated that, in the

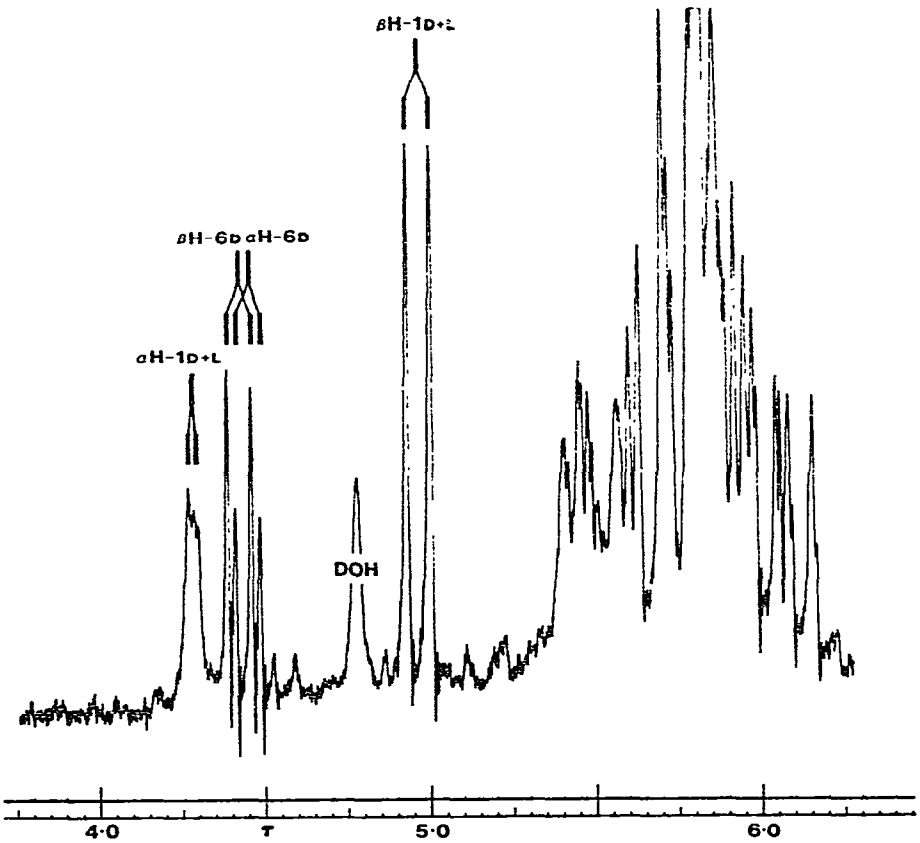

Fig. 1. P.m.r. spectrum (100 MHz) in D_2O of the reaction products from a D-galactose oxidase-treated mixture of 49.5% of D- and 50.5% of L-galactose.

favoured rotamer, the dihedral angle of H-6 and H-5 was ~90°. Although the presence of methoxyl groups at positions 1–3 of the galactopyranose residue did not hinder the enzyme reaction, substituents at C-4 were inhibitory[16].

The utility of this enzyme reaction to determine the enantiomeric composition of mixtures of D- and L-galactose was assessed by treating synthetic mixtures with D-galactose oxidase and analysing the p.m.r. spectra of the products. Integration of the signals for the anomeric and aldehydrol protons gave inconsistent results, due to background fluctuations and the proximity of the peaks. Determination of the area by weighing the excised peaks proved to be the most accurate method, and the percentage of D-galactose was calculated from the weights of the aldehydrol signals (H-6α+H-6β) from the oxidised D enantiomer, and those of the anomeric signals (H-1α+H-1β) from the L enantiomer and the oxidised D form, *cf.* Fig. 1. The data so obtained (Table I) were accurate to within ±4.5% (mean deviation, 2.18%).

The doublets for H-1α in the spectra of most of the galactose derivatives, before and after enzyme treatment, were not well-resolved, because of virtual long-range

TABLE I

DETERMINATION OF D-GALACTOSE, ALONE AND IN MIXTURES OF D- AND L-GALACTOSE, BY THE ENZYME–P.M.R.-SPECTROSCOPIC PROCEDURE

D-*Galactose in* D,L *mixture* (%)	*Percentage of* D-*galactose*	
	Area $(\alpha+\beta)$*H-6* (D) × *100* / *Area* $(\alpha+\beta)$*H-1* (D+L)	*Area* $(\alpha+\beta)$*H-6* (D) × *63.2* / *Area H-1*β (D+L)
0	0	0
5.5	8.3	7.6
10.4	13.5	12.1
24.6	23.6	22.1
43.1	42.0	40.4
49.5	53.3	50.1
50.0	48.0	45.7
66.0	64.0	62.5
78.2	78.7	76.2
86.4	84.1	88.0
87.1	91.0	85.9
94.7	90.2	93.7
100.0	101.4	101.1

coupling effects. An alternative calculation for the percentage of D-galactose in the oxidised, synthetic mixtures of D and L forms involved the areas of the aldehydrol peaks, the well-defined signal for H-1β, and a constant that was derived from the relationship shown in Fig. 2. The percentage of D-galactose determined by this alternative method had slightly increased accuracy (Table I), with a mean deviation of 1.8%.

The hydrolysate from the sulphated galactan from *Rhodymenia pertusa* was subjected to analysis by the foregoing procedure and also the fermentation technique with yeast. When 3–5-day fermentation of the hydrolysate with D-galactose-adapted *Saccharomyces cerevisiae* was employed, coupled with determination of the carbohydrate content before and after fermentation and allowing for the contents of 6-*O*-methyl-D-galactose and 3,6-anhydrogalactose, average contents of 19.7% of L- and 43.8% of D-galactose were obtained for the polysaccharide. However, the reproducibility of the results was poor. The D-galactose oxidase–p.m.r. procedure provided reproducible results, and average contents of 18.6% of L- and 44.9% of D-galactose. The enzyme procedure was free from the problems associated with that involving adapted micro-organisms, was accurate to within ±5%, and was more rapid, reproducible, and convenient than the fermentation method.

EXPERIMENTAL

General. — P.c. was performed on Whatman No. 1 paper with *A*, 10:4:3 ethyl acetate–pyridine–water; *B*, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water;

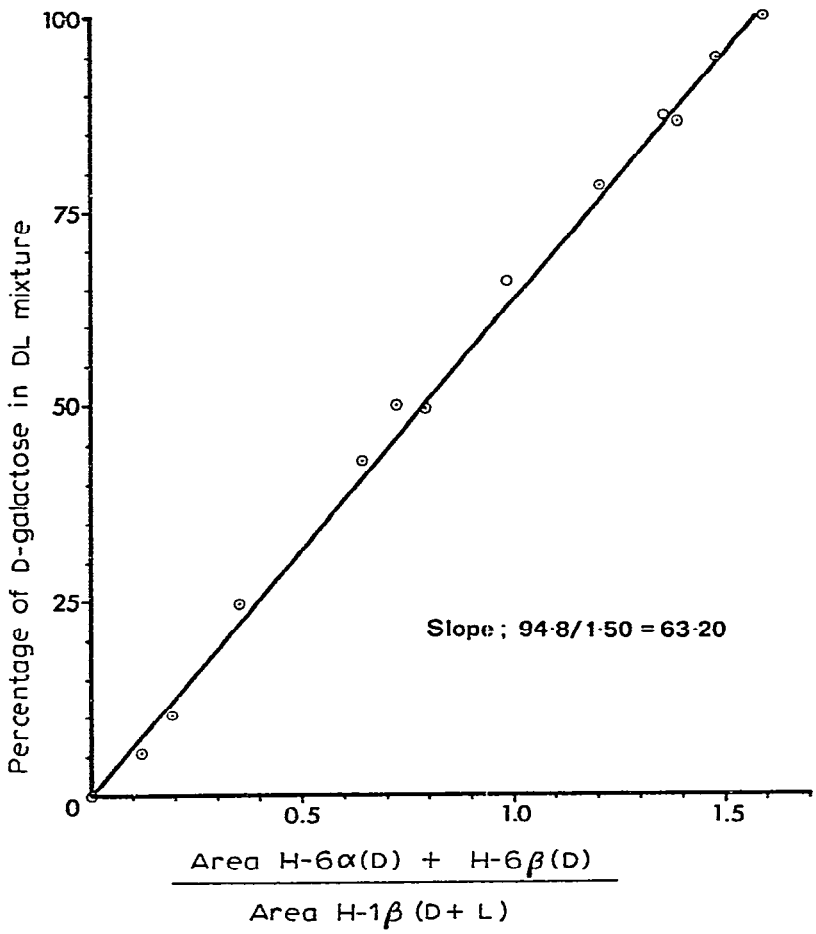

Fig. 2. Relationship between the actual D-galactose in the synthetic mixtures and the ratio of the areas of the signals for the aldehydrol and β-anomeric protons.

C, 3:1:1 1-butanol–ethanol–water; and detection with alkaline silver nitrate[23]. Total sugar was determined by the phenol–sulphuric acid method[24]; and 3,6-anhydro-galactose by the resorcinol–hydrochloric acid method[25], with methyl 3,6-anhydro-α-D-galactoside[26] as the standard for calibration. G.l.c. was performed on a column (1.20 m × 6 mm) of 0.2% of poly(glycol adipate), 0.2% of poly(glycol succinate), and 0.4% of XF-1150 on chlorodimethylsilane-treated Chromosorb W (60–80 mesh), with a nitrogen flow-rate of 60 ml/min at 150°. P.m.r. spectra were obtained on solutions in D_2O at 28°, unless otherwise stated, using a Varian HA-100 spectrometer; τ values are relative to tetramethylsilane as external standard.

L-Galactose, $[\alpha]_D^{20}$ −80° (*c* 1, water), was prepared from D-galacturonic acid by reduction to L-galactonic acid with sodium borohydride, followed by reduction with sodium amalgam[27]. The sulphated galactan {$[\alpha]_D^{20}$ +30° (*c* 1, water); Found: total

carbohydrate, 68.6%; anhydro-3,6-anhydrogalactose, 1.8%; sulphate ester content (as SO_3Na^-), 29.5%} was isolated from *Rhodymenia pertusa* by extraction with hot water, fractionated twice with hexadecyltrimethylammonium bromide[20], and purified by treatment with trichloroacetic acid. On hydrolysis, the polysaccharide gave only galactose and 6-*O*-methylgalactose, as shown by p.c. (solvents *A–C*), and g.l.c. of the derived alditol acetates[28] indicated proportions of 19.1:1.

D-Galactose oxidase (*Dactylium dendroides*, EC 1.1.3, Worthington Biochemical Corp.) was used as a solution in deionised water containing ~85 units/ml. Portions (1 ml) were syringed into Pierce Hypo-Vials, flushed with nitrogen, sealed with septa, and stored at −31° until required. Catalase (beef liver, EC 1.11.1.6, Worthington Biochemical Corp.) was stored at ~5° as a solution of ~35,000 units/ml. *Saccharomyces cerevisiae* was adapted by transitional culturing from a D-glucose to a D-galactose culture-medium.

Standard enzyme-reaction procedure. — A sample of the sugar or sugar mixture (≯100 mg) was dissolved in oxygen-saturated, distilled, deionised water (1–5 ml) to give ~0.1M concentration of the D-galactose moiety. Catalase solution (0.3 ml, ~10,500 units) was added by syringe, followed by D-galactose oxidase solution (1 ml, ~85 units). The flask was flushed with oxygen, sealed, and stirred slowly, without vortexing. After 20 h, the mixture was concentrated to a syrup, which was treated thrice with deuterium oxide (99.98%) prior to p.m.r. spectroscopy.

Enzymic dehydrogenation of standard sugars. — D-Galactose (100 mg) was oxidised by the standard procedure, and for 24 h the reaction was monitored by p.c. (solvent *A*). The oxidation of D-galactose [τ 4.28 ($J_{1,2}$ 2.7 Hz, H-1α) and 4.97 ($J_{1,2}$ 7.2 Hz, H-1β)] to *meso-galacto*-hexodialdose, R_{GAL} 0.54 (solvent *A*) [τ 4.28 ($J_{1,2}$ ~2.6 Hz, H-1α), 4.97 ($J_{1,2}$ 7.2 Hz, H-6β), 4.43 ($J_{5,6}$ 7.1 Hz, H-6β), and 4.46 ($J_{5,6}$ 7.1 Hz, H-6α)] was complete in 18–20 h.

L-Galactose (100 mg), when treated with the enzyme mixture, afforded no oxidation product, as shown by p.c. (solvents *A–C*), and the p.m.r. spectrum remained unchanged [τ 4.28 ($J_{1,2}$ ~2.7 Hz, H-1α) and 4.97 ($J_{1,2}$ 7.2 Hz, H-1β)].

Methyl α-D-galactopyranoside[29] [100 mg, τ 4.70 ($J_{1,2}$ ~2.8 Hz, H-1)] afforded methyl α-D-*galacto*-hexodialdo-1,5-pyranoside [τ 4.69 ($J_{1,2}$ ~2.8 Hz, H-1) and 4.42 ($J_{5,6}$ 7.2 Hz, H-6)], and methyl β-D-galactopyranoside[29] [100 mg, τ 5.22 ($J_{1,2}$ 7.2 Hz, H-1) yielded methyl β-D-*galacto*-hexodialdo-1,5-pyranoside [τ 5.21 ($J_{1,2}$ 7.2 Hz, H-1) and 4.39 ($J_{1,2}$ 7.2 Hz, H-6)], when treated with the enzyme mixture under the standard conditions.

Methyl 2,3-di-*O*-methyl-α-D-galactopyranoside[30,31] [τ 4.45 ($J_{1,2}$ ~2.8 Hz, H-1)] yielded methyl 2,3-di-*O*-methyl-α-D-*galacto*-hexodialdo-1,5-pyranoside [τ 4.45 ($J_{1,2}$ ~2.8 Hz, H-1) and 4.41 ($J_{5,6}$ 7.2 Hz, H-6); in $CDCl_3$: τ 5.03 ($J_{1,2}$ 2.7 Hz, H-1) and 0.37 (s, H-6)], and methyl 2,3-di-*O*-methyl-β-D-galactopyranoside[30,32] (τ 5.17, $J_{1,2}$ 7.2 Hz, H-1) yielded methyl 2,3-di-*O*-methyl-β-D-*galacto*-hexodialdo-1,5-pyranoside [τ 5.17 ($J_{1,2}$ 7.2 Hz, H-1) and 4.40 ($J_{5,6}$ 7.2 Hz, H-6)], when treated with D-galactose oxidase according to the standard procedure.

Determination of the D *enantiomer in mixtures of* D- *and* L-*galactose.* — Samples

of mixtures (50–100 mg) of D- and L-galactose, containing 0–100% of one enantiomer, were oxidised by the standard enzyme procedure, and the subsequent p.m.r. spectra (500-Hz sweep-width) were analysed. The sides of the peaks for H-1α(D) and/or H-1α(L), H-1β(D) and/or H-1β(L), H-6α(D), and H-6β(D) were extended to the baseline, and then excised and weighed.

The percentage of D-galactose was calculated by the formula [weight ($\alpha+\beta$)H-6(D) × 100]/[weight ($\alpha+\beta$)H-1(D+L)] to afford the values in Table I. A plot (Fig. 2) of the percentage of D-galactose in the D,L mixture *versus* the ratio [weight ($\alpha+\beta$)H-6(D)]/[weight H-1β(D+L)] gave a slope with the constant $K = 63.20$, which was used in the alternative calculation of the percentage of D-galactose, namely, [weight ($\alpha+\beta$)H-6(D) × K]/[weight H-1β(D+L)]. Routinely, several copies of the spectra were prepared to provide mean values. For a mixture of 43.1% of D- and 56.9% of L-galactose, the estimated percentage of D-galactose by peak area (weight) determinations were 42.0, 41.8, 42.9, 42.4, 41.6, and 41.3% (average of 41.9 ±1%).

Determination of the percentage of D- *and* L-*galactose in the galactan from* Rhodymenia pertusa. — (*a*) *By fermentation with* Saccharomyces cerevisae. The polysaccharide (400 mg) was hydrolysed with 0.5M sulphuric acid in a sealed tube at 100° overnight, conditions under which the anhydrogalactose was degraded. The hydrolysate was neutralised ($BaCO_3$), filtered, deionised with Rexyn 101 (H^+) and Duolite A4 resins, and concentrated to dryness. The residue was dissolved in water (25 ml), and 4 aliquots (5 ml) were separately shaken under sterile conditions with washed D-galactose-adapted *S. cerevisiae* cells (5 ml) for 3 days. The yeast cells were then removed by centrifugation, and three of the supernatants were made up to 200 ml and analysed for total carbohydrate. The remaining supernatant was treated with an additional portion of yeast cells for a further 2 days, and the remaining carbohydrate was assessed as described above. P.c. (solvents *A–C*) of the supernatants revealed galactose only. The total carbohydrate assessed prior to fermentation was 40.46 mg, and after fermentation, values of 10.27, 13.35, and 11.25 mg were obtained, corresponding to 25.4, 33.0, and 27.8% of L-galactose. After two fermentations, a value of 12.90 mg (31.9%) was obtained. The average L-galactose content of the hydrolysate was 29.5%, which indicated the contents of L- and D-galactose of the galactan to be 19.7 and 43.8%, respectively, allowing for contents of 3.3% of 6-*O*-methylgalactose and 1.8% of 3,6-anhydrogalactose.

(*b*) *By the enzyme–p.m.r.-spectroscopy procedure.* The galactan (100 mg) was hydrolysed as in (*a*), and oxygen-saturated water (3.6 ml) was added to the product followed by catalase (0.3 ml) and D-galactose oxidase (1 ml). After 20 h, the solution was analysed in duplicate by p.m.r. spectroscopy. The percentage of D-galactose, determined by the alternative formula described above, was as follows: spectrum 1, 67.7, 66.6, 68.9%; spectrum 2, 68.8, 66.3, 64.8% (average 67.2%). It follows that the contents of D- and L-galactose in the galactan were 44.9 and 18.6%, respectively, taking into account the content of other sugars as in (*a*).

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