278. *A Preliminary Investigation of Galactogen from the Albumin Glands of* Helix pomatia.

By E. Baldwin and D. J. Bell.

Galactogen, isolated from the albumin gland of *Helix pomatia*, has been methylated to a methoxyl content of 43%. Hydrolysis of the methylated galactogen yielded approximately equimolecular proportions of 2 : 3 : 4 : 6-tetramethyl and 2 : 4-dimethyl *d*-galactopyranose. No evidence could be obtained for the presence of sugars other than *d*-galactose in galactogen.

Whereas a considerable number of polysaccharides of plant origin are known, the occurrence of only four has been definitely established among animals. These are glycogen (very widely distributed), chitin (especially among *Arthropoda*), cellulose (probably con-

fined to *Tunicata*), and lastly, the polysaccharide first obtained from the albumin glands of *Helix pomatia* and described under the name of " tierisches Sinistrin " by Hammarsten (*Pflüger's Arch.*, 1885, **36**, 373). This substance, and a seemingly identical product obtained from the eggs of the same snail, was subsequently studied by May (*Z. Biol.*, 1931, **91**, 215; 1932, **92**, 319, 325; 1934, **95**, 277, 401, 606, 614), who concluded that on acid hydrolysis it yields *d*-galactose as sole product, and accordingly proposed the name " galactogen."

Since May's chemical investigations covered only a few general properties of the polysaccharide, we considered it desirable to proceed along conventional lines with a view to establish, if possible, the chemical structure of galactogen. Knowledge of this is of particular interest in relation to metabolic experiments by one of us (Baldwin, *Biochem. J.*, 1938, **32**, 1225). While our investigations were in progress, Schlubach and Loop (*Annalen*, 1937, **532**, 228) described the methylation of galactogen isolated from the whole bodies of snails. Prepared in this way, the galactogen must be separated from a large proportion of glycogen, and we have preferred the more direct method of using isolated albumin glands as our starting material, since these contain all the galactogen of the body and are at the same time free from glycogen (May, *Z. Biol.*, 1934, **95**, 401). Hydrolysis of the methylated product obtained by Schlubach and Loop yielded equimolecular proportions of unidentified tetra- and di-methyl galactoses, and we have continued research in this field in order to obtain more definite information.

We isolated our galactogen by subjecting dried albumin glands to digestion with 30% aqueous potassium hydroxide, following the procedure of Bell and Young (*Biochem. J.*, 1934, **28**, 882) for glycogen. Purification by precipitation with alkaline copper sulphate (see below) and subsequent decomposition of the copper–galactogen complex gave an identical product. After careful drying, our material had the following properties (corrected for ash content, 3·2%): (*a*) $[\alpha]_D^{20}$ — 16·1° (water; $c = 1{\cdot}0$, $l = 2$); Schlubach and Loop (*loc. cit.*) found — 17·6°, and, like ourselves, failed to observe the higher value of — 22·7° recorded by May (*Z. Biol.*, 1932, **92**, 325), but it should be pointed out that May's figure was obtained for galactogen isolated from the eggs of the snail, whereas, using material prepared from the whole body, he found — 13·5° (*ibid.*, 1931, **91**, 215); (*b*) " reduction," 0·57 (Macleod–Robison; glucose = 100); (*c*) organic phosphorus, 0·2% (inorganic phosphorus, nil); (*d*) faintly opalescent solutions were formed in water, the opalescence markedly increasing on the addition of salts; (*e*) the material gave no colour on addition of iodine, was free from pentoses and uronic acids, and readily formed a precipitate on boiling with May's alkaline copper reagent (*ibid.*, 1931, **95**, 277).

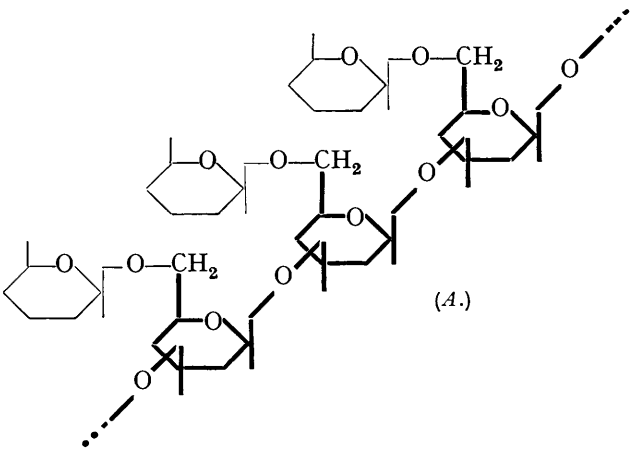
May repeatedly observed that, when galactogen was hydrolysed by dilute acids, the final $[\alpha]_D$ of the solution was + 53·6° instead of + 80·5° for the equilibrium mixture of α- and β-*d*-galactoses. Since his determinations of the reducing power of the hydrolysate indicated that complete hydrolysis had taken place, and since he obtained the expected yields of mucic acid on oxidation, May concluded that he had to deal with a final hydrolysis product consisting of β-*d*-galactose ($[\alpha]_D$ = + 53°) in some stabilised form. He was able, nevertheless, to isolate and identify crystalline *d*-galactose from the products of hydrolysis, and the behaviour of this sugar was normal. The anomalous behaviour exhibited by galactogen on acid hydrolysis has been fully confirmed in the present work, and the isolation and identification of crystalline *d*-galactose was also effected. May's explanation of the final $[\alpha]_D$ did not seem probable; and it appeared possible that the discrepancy might be due to the presence in the hydrolysates of *l*-galactose, which is known to occur naturally (Winterstein, *Ber.*, 1898, **31**, 1571; Oshima and Tollens, *Ber.*, 1901, **34**, 1421; v. Lippmann, *Ber.*, 1922, **55**, 3038; Anderson, *J. Biol. Chem.*, 1933, **100**, 249), or of some other lævorotatory substance. Our attempts to detect material of this kind have failed, and we have provisionally to suppose, with Schlubach and Loop, that " reversion products " may arise from *d*-galactose in the course of hydrolysis under the conditions employed; those authors have obtained much higher final $[\alpha]_D$ values by submitting galactogen to hydrolysis by 40% hydrochloric acid at 0°.

In agreement with Schlubach and Loop, we found acetylation very difficult, but we ultimately obtained a triacetate in 80% yield. After several attempts, it was found

possible to introduce 25·6% of methoxyl into the polysaccharide by a single process of de-acetylation and simultaneous methylation: direct methylation of the free polysaccharide was not practicable. Thirteen further methylations raised the methoxyl content to 43·1% (91% yield; $[\alpha]_D^{22} = -20°$, in water; $c = 3$, $l = 2$).

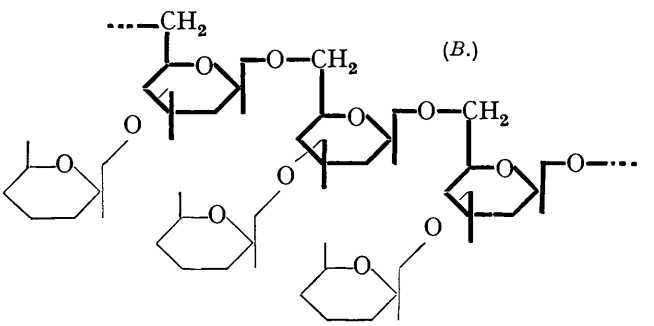
Hydrolysis of the methylated product yielded approximately equimolecular proportions of 2 : 3 : 4 : 6-tetramethyl *d*-galactose (identified as the crystalline anilide) and a crystalline dimethyl *d*-galactose, identified as the 2 : 4-derivative (previously obtained from another source in the Birmingham laboratory; private communication from Dr. F. Smith). This therefore constitutes a further instance in which galactose units are linked through C_3 (cf. Percival and Somerville, J., 1937, 1615; Hirst and Jones, private communication). Owing to lack of material, no other methylated sugars have yet been detected.

The results suggest that at least the greater part of galactogen possesses a hitherto unknown type of structure, and may be formulated in one of two ways: (*A*) a chain of



(*A*.)

d-galactopyranose units linked 1 : 3 (probably in the β-configuration in view of the change of rotation observed on acid hydrolysis), each unit bearing as side chain a single *d*-galactopyranose unit, glycosidically linked at C_6; (*B*) a chain of *d*-galactopyranose units united 1 : 6, with the side chains attached at C_3. Both these formulæ admit of an "end



(*B*.)

group." In (*A*) this would be expected to give rise to 2 : 3 : 4-trimethyl, and in (*B*) to 2 : 4 : 6-trimethyl *d*-galactose, and we intend to search for such an end group later in the year, when the physiological condition of *H. pomatia* is again suitable; at present (July), owing to egg-laying, the animals are out of season for culinary purposes, and the albumin glands are depleted of galactogen.

A closed loop of galactose units is a third possibility, and in this case an "end group" would not exist.

Experimental.

Isolation and Properties of the Galactogen.—The albumin glands were dissected out and immediately placed in alcohol. After hardening, they were ground, and dried at 90° until the weight was constant. The dry material was then dissolved in approximately 2 parts (wt./vol.) of aqueous 30% potassium hydroxide and heated at 100° for 3 hours. To the resulting dark brown solution, water (1 vol.) was added, followed by 2 vols. of alcohol. The sticky brown precipitate was dissolved in 30% potassium hydroxide and heated at 100° for a further 2 hours. The crude galactogen was then precipitated by alcohol as before, dissolved in water, the alkali neutralised by acetic acid, and the solution centrifuged. Four precipitations by acetic acid (cf. Bell and Young, *loc. cit.*) were then carried out, and the final product was obtained as a white powder (yield 30% of dry weight of the glands), easily soluble in water, and displaying the properties already mentioned after drying at 110°/0·05 mm.

Hydrolysis by 1% hydrochloric acid. A 0·97% solution of galactogen in 1% hydrochloric acid solution gave the following rotations (calculated in terms of galactogen and corrected for 3% of ash) in the course of hydrolysis at 100° :

Time (mins.)	0	15	30	45	75	105	150	210	270
$[\alpha]_D$	−10·6°	+3·7°	+18·1°	+26·9°	+40·4°	+49·4°	+56·8°	+62·1°	+62·7°

The final $[\alpha]_D$ in terms of galactose was + 56·5°.

For investigation of the products of hydrolysis, samples of approximately 1% solutions of the polysaccharide in 1% hydrochloric acid were heated at 100° for 5—6 hours and then neutralised. The following data are corrected for ash content.

Reducing power (Macleod and Robison) 89·8 (galactose = 100); (Hagedorn and Jensen) 85·8 (galactose = 100).

Mucic acid (van der Haar, *Biochem. Z.*, 1917, **81**, 263) 90·8%. Galactose phenylmethylhydrazone (Pirie, *Biochem. J.*, 1936, **30**, 369) : from 1·96 g. of galactogen we obtained 2·45 g. of this hydrazone (yield 70%), m. p. 178°; m. p. after recrystallisation from boiling water, 189°, unchanged by mixture with an authentic sample.

Fermentation. 2·65 G. of polysaccharide, after hydrolysis and fermentation with galactose-trained yeast (Stephenson and Yudkin, *Biochem. J.*, 1936, **30**, 506), left only a negligible amount of unidentifiable material.

Acetylation. 10 G. of galactogen were dissolved in 60 ml. of water and precipitated by adding 120 ml. of alcohol. The precipitate was centrifuged, and added while still moist to 200 ml. of pyridine, which caused it to swell. 200 Ml. of acetic anhydride were then added, the whole was kept for 12 hours at room temperature, and then for 4 hours at 70°. Mechanical stirring was maintained throughout the preparation. The product was isolated by pouring the reaction mixture into excess of water and centrifuging the white precipitate. This was then treated again with the acetylating reagents as described above. The final product was a white powder (14 g.), insoluble in all solvents except, with difficulty, in acetone (Found : $CO{\cdot}CH_3$, 41·0, 42·5%).

Methylation. 10 G. of the acetate were ground with 200 ml. of acetone to give a turbid solution. This was warmed to 50° with mechanical stirring, and 8 lots each of 20 ml. of methyl sulphate and 80 ml. of 30% potassium hydroxide (introduced in that order) were added every 2 minutes. Subsequently, 8 lots each of 10 ml. of the ester and 25 ml. of the 30% alkali were added every 10 minutes. On boiling off the acetone, no precipitation was observed; heating at 100° was continued for 45 minutes, and the solution was then cooled, neutralised with acetic acid, and dialysed for 24 hours against a rapid stream of tap-water passing through a specially designed cellophane immersion apparatus. This method avoids the danger of high osmotic pressure within the sac, and is eminently suitable for the rapid removal by dialysis of considerable quantities of salts. The dialysed solution was evaporated to dryness in a vacuum, the residue dissolved in very dilute acetic acid, the solution dialysed in cellophane sacs against several changes of distilled water, and finally again evaporated to dryness in a vacuum. The residue (7·5 g.) was a scaly solid having a methoxyl content of 25·6%. This product was dissolved in the minimum of 30% potassium hydroxide, stirred at 50°, and 40 ml. of methyl sulphate and 130 ml. of 30% alkali were added in one-tenth portions at 10-minute intervals. As solid material began to separate after the first few additions, acetone was added to promote solution. After the reaction mixture had been worked up in the usual way, the crude product separating from the hot liquors was dissolved in acetone and again methylated (yield 7·8 g.) (Found : OMe, 37·4%). Seven further methylations were carried out, making 10 in all. The crude product,

purified by precipitation from acetone solution by light petroleum, was a white powder (6·5 g.), soluble in cold water, acetone, and chloroform (Found : OMe, 42·5%). Four further methylations raised the methoxyl content by only 0·6% (Found : OMe, 43·1%).

Hydrolysis of Methylated Galactogen : Separation of the Cleavage Products.—6·0 G. of methylated galactogen (OMe, 43%) were dissolved in 100 ml. of concentrated hydrochloric acid, the solution saturated with hydrogen chloride at — 15°, and kept for 2 hours (cf. Schlubach and Loop, *loc. cit.*). After removal of hydrogen chloride by aspiration at room temperature, the solution was concentrated in a vacuum to half its bulk, neutralised with lead carbonate, completely decolorised by filtration through charcoal, and evaporated to dryness in a vacuum. The residue was extracted with ethyl acetate–acetone (1 : 1), and evaporation of this solution gave 5·3 g. of a colourless syrup. This was boiled with 1% methyl-alcoholic hydrochloric acid until non-reducing (3 hours), and after neutralisation with silver carbonate and evaporation to dryness, the resulting syrup was fractionally distilled in a high vacuum through a vacuum-jacketed fractionating column. The following fractions were ultimately obtained : (i) 1·86 g., b. p. 106—112°/0·07 mm., $n_D^{18°}$ 1·4495 ($n_D^{18°}$ of last drop 1·4490), OMe, 60·8%; (ii) 0·14 g., b. p. 112—120°/0·07 mm., $n_D^{18°}$ 1·4500 ($n_D^{18°}$ of last drop 1·4515), OMe, 60·5%; (iii) 0·45 g., b. p. 120—150°/0·07 mm., $n_D^{18°}$ 1·4585 ($n_D^{18°}$ of last drop 1·4655), OMe, 54·3%; (iv) 2·06 g. (crystalline), b. p. 150—170°/0·05 mm., $n_D^{18°}$ of fused crystals 1·4785, OMe, 41·0%; (v) residue, 0·5 g. (crystalline), OMe, 41·1%.

Examination of the Cleavage Products : Identification of 2 : 3 : 4 : 6-*Tetramethyl and* 2 : 4-*Dimethyl* d-*Galactose.*—Fractions (i) and (ii) were tetramethyl methylhexoside. Hydrolysis by aqueous acid yielded a tetramethyl hexose identified as 2 : 3 : 4 : 6-tetramethyl *d*-galactopyranose by conversion into the crystalline anilide in the anticipated yield; m. p. 195—196°, unchanged on admixture with an authentic sample. Fraction (iii), which was too small to permit of an accurate examination, appeared to consist of about 65% pentamethyl hexose and 35% trimethyl hexose, provided tetramethyl hexose was absent; (iv) and (v) were dimethyl methylhexoside, and clearly resembled the mixture of dimethyl α- and β-methyl-*d*-galactosides obtained by Schlubach and Loop. Methylation followed by hydrolysis yielded 2 : 3 : 4 : 6-tetramethyl *d*-galactose (identified as the anilide). Hydrolysis of the crystals with aqueous acid yielded a syrup which rapidly crystallised from moist ethyl acetate containing a little alcohol; m. p. 100—103°; mixed m. p. of specimen, m. p. 98°, with authentic 2 : 4-dimethyl *d*-galactose hydrate (carried out by Dr. F. Smith at Birmingham) showed no depression; $[\alpha]_D^{20°}$ (at equilibrium) + 85·7° (in water) (Found : for anhydrous material, C, 46·0; H, 7·6; OMe, 29·0. Calc. for $C_8H_{16}O_6$: C, 46·2; H, 7·7; OMe, 29·8%). The dimethyl sugar, when treated with phenylhydrazine and acetic acid, yielded a monomethyl osazone (Found : C, 59·4; H, 6·53; OMe, 8·4; N, 14·5. Calc. for $C_{19}H_{24}O_4N_4$: C, 61·3; H, 6·45; OMe, 8·33; N, 15·06%). Recrystallised from aqueous alcohol, this substance melted at 148—150°; mixed m. p.'s with specimens of 3-methyl *d*-galactosazone (Robertson and Lamb, J., 1934, 1321) and 6-methyl *d*-galactosazone (Freudenberg and Smeykal, *Ber.*, 1926, **59**, 100) showed considerable depression. The elimination of a methyl group from position 2 in galactose has already been shown to take place in analogous instances by Robertson and Lamb (*loc. cit.*), Percival and Ritchie (J., 1936, 1765), Percival and Somerville (*loc. cit.*), and Oldham and Bell (*J. Amer. Chem. Soc.*, 1938, **60**, 323). The osazone appears to be identical with the 4-methyl *d*-galactosazone described by Percival and Ritchie (*loc. cit.*), and was thus derived from 2 : 4-dimethyl *d*-galactose. Confirmatory evidence was obtained by oxidising the sugar to the corresponding acid, followed by lactonisation under conditions favourable to the formation of γ-lactones. The product (Found : OMe, 29·6; 0·0590 g. required 5·68 ml. of N/20-NaOH. Calc. for $C_8H_{14}O_6$: OMe, 30·09%; N/20-NaOH, 5·72 ml.) when dissolved in water displayed polarimetric behaviour typical of a δ-galactonolactone, *viz.*,

Time, mins.	5	25	55	115	415	24 hrs.	96 hrs.
$[\alpha]_D^{25°}$ (*c*, 2·95; *l*, 2)	+105°	+100°	+91·5°	+77·2°	+48·8°	+46·8°	+46·8°

Treated with methyl-alcoholic ammonia in the usual manner, the lactone yielded a crystalline amide (prisms from acetone–alcohol, 1 : 1; m. p. 165° after softening at 163°) which gave a negative Weerman reaction (cf. Ault, Haworth, and Hirst, J., 1934, 1722), confirming the presence of a methoxyl group at C_2. A mixed m. p., determined by Dr. F. Smith, with authentic 2 : 4-dimethyl *d*-galactonamide showed no depression (Found : C, 42·9; H, 7·8; N, 6·5; OMe, 27·5. Calc. for $C_8H_{17}O_6N$: C, 43·05; H, 7·6; N, 6·27; OMe, 27·8%).

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