451. *Amino-sugars and Related Compounds. Part IV.* Isolation and Properties of Oligosaccharides obtained by Controlled Fragmentation of Chitin.†*

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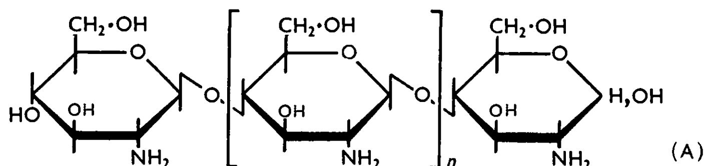
Graded acidic hydrolysis of chitosan (obtained by alkaline deacetylation of chitin) followed by selective *N*-acetylation of the fragments yields a mixture of saccharides of which the first seven members have been isolated by chromatography. Evidence is presented which suggests that the saccharides constitute a polymer-homologous series. Structural information on the di- and tri-saccharides is provided by periodate oxidation.

Nomenclature.—Chitin is a substituted polysaccharide since it contains acetylated amino-groups and the logical nomenclature for saccharides derived from chitin should be based on the unsubstituted (*i.e.*, de-*N*-acetylated) polysaccharide. De-*N*-acetylated chitin is widely referred to in the literature as chitosan and it is therefore suggested that the saccharides represented by the general formula (A) be termed chitosaccharides, *e.g.*, chitobiose ($n = 0$), chitotetraose ($n = 2$), etc.



The saccharides containing one acetyl group on each nitrogen atom, which are the true fragments of chitin, may then be described as tri-*N*-acetylchitotriose, penta-*N*-acetylchitopentaose, etc.

The description of non-uniformly substituted saccharides would require that the constituent monosaccharide units of the oligosaccharides be numbered from the reducing moiety (or modification thereof) so that substituents could be described (*a*) in the accepted manner to indicate their nature and point of attachment to a monosaccharide unit, and (*b*) by a superscript numeral to indicate the position, in the oligosaccharide chain, of the monosaccharide unit to which the substituent is attached. Thus partial *N*-acetylation of chitotriose might yield di-$N^{1,3}$-acetylchitotriose. This system is capable of general extension to chitosaccharide derivatives.

A possibility of confusion would be eliminated if the use of the trivial name chitose for 2 : 5-anhydro-D-mannose were discontinued.

Degradation of chitin by various methods has yielded the following characterised fragments: 2-acetamido-2-deoxy-D-glucose,[1] 2-amino-2-deoxy-D-glucose hydrochloride,[2] di-*N*-acetylchitobiose,[3] chitobiose octa-acetate,[4] and chitotriose hendeca-acetate.[4] Recently Horowitz *et al.*[5] reported that the graded acidic hydrolysis of chitosan (de-*N*-acetylated chitin) yields a series of saccharides which may be fractionated by elution from ion-exchange resins. Only the disaccharide (chitobiose) of this series was isolated and

* Part III, *J.*, 1958, 1890.

† A preliminary report of some of these results has been given in *Chem. and Ind.*, 1957, 208.

1 Fränkel and Kelly, *Monatsh.*, 1902, **23**, 123.

2 Ledderhose, *Ber.*, 1876, **9**, 1200.

3 Zilliken, Braun, Rose, and György, *J. Amer. Chem. Soc.*, 1955, **77**, 1296.

4 Zechmeister and Tóth, *Ber.*, 1931, **64**, 2028; 1932, **65**, 161.

5 Horowitz, Blumenthal, Seppala, and Roseman, *Fed. Proc.*, 1955, **14**, 229; Horowitz, Blumenthal, and Roseman, *J. Amer. Chem. Soc.*, 1957, **79**, 5046.

characterised (as octa-acetate). We now report a partial degradation of chitin which permits the isolation of a series of *N*-acetylated chitosaccharides up to at least the hepta-saccharide.

The chitin used was isolated from the shell of the edible crab, *Cancer pagurus*. Insolubility complicates the controlled fragmentation. Preliminary attempts, by dissolution in strong mineral acid (*e.g.*, concentrated hydrochloric acid saturated at 0° with hydrogen chloride, concentrated sulphuric acid), were unsatisfactory because of the large excess of acid required; concentrated sulphuric acid caused extensive sulphation.

Our acetolysis [6] of chitin gave only chitobiose octa-acetate (in low yield), although it was later shown that tri-*N*-acetylchitotriose with pyridine and acetic anhydride yielded a crystalline product.

Catalytic de-*O*-acetylation (Zemplén) of chitobiose octa-acetate and 2-acetamido-1 : 3 : 4 : 6-tetra-*O*-acetyl-2-deoxy-α- and -β-D-glucose afforded in each case a mixture of at least five components, revealed by paper chromatography. The result was similar when 2-acetamido-2-deoxy-D-glucose was treated under the de-*O*-acetylation conditions. The artefacts in each case had R_F values greater than that of the saccharide from which they were formed. Comparable results have been reported by Zilliken *et al.*[3]

Multiple fractionation, on charcoal–Celite,[7] of the complex mixture obtained by de-*O*-acetylation of a mixture of acetylated lower chitosaccharides, isolated after acetolysis of chitin, gave crystalline di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose. However, the low overall yields and tedious isolation largely deprive the acetolysis method of preparative value.

In a parallel fractionation of the products obtained on catalytic de-*O*-acetylation of a mixture of acetylated chitosaccharides, one of the artefacts (*A*) was isolated crystalline in very low yield. Analysis suggested that it was derived from 2-acetamido-2-deoxy-D-glucose by loss of water. The strong infrared absorptions at 1637 and 1545 cm.$^{-1}$ indicated that the acetamido-group was intact. In these respects and in R_F value (in water-saturated butan-1-ol), the product *A* resembles chromogen I isolated by Kuhn and Krüger [8] from a mixture of three chromogens (believed to be those involved in the Morgan–Elson test [9,10]) obtained by treatment of 2-acetamido-2-deoxy-D-glucose with a strongly basic resin or barium hydroxide at 90° or by treatment of 2-amino-2-deoxy-D-glucose penta-acetate with barium methoxide at room temperature. Chromogen I, which has not been obtained crystalline and is probably formed by dehydration of 2-acetamido-2-deoxy-D-glucofuranose,[8] reacts strongly with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) in acid solution to yield an intense colour with absorption maxima at 512 (weak), 542, and 584 mμ. Under the same conditions product *A* gave only a weak fading colour but after treatment with alkali, as in the Morgan–Elson test, and subsequent reaction with Ehrlich's reagent an intense colour was produced with absorption maxima at 512 (weak), 543, and 582 mμ, similar to that given by chromogen I. Chromogen I showed weak absorption at 230 mμ, whereas product *A* showed no maximal absorption in the range 210—260 mμ, and, further, the affinities of the two compounds for charcoal–Celite were different: chromogen I was eluted only with >15% aqueous alcohol (*i.e.*, 15% ethanol v/v) whereas product *A* was eluted with 5%. Although the structural relations between product *A* and chromogen I cannot be precisely defined the former is possibly the precursor of the latter.

Chitin is de-*N*-acetylated by solid potassium hydroxide at 170—190° under nitrogen.[11] The product, chitosan, usually isolated as a water-soluble salt which still contains *N*-acetyl residues, is more amenable to controlled fragmentation than chitin itself. Chitosan

[6] Bergmann, Zervas, and Silberkweit, *Ber.*, 1931, **64**, 2436.
[7] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[8] Kuhn and Krüger, *Chem. Ber.*, 1956, **89**, 1473; 1957, **90**, 264.
[9] Morgan and Elson, *Biochem. J.*, 1934, **28**, 988.
[10] Aminoff, Morgan, and Watkins, *ibid.*, 1952, **51**, 379.
[11] Ricketts, *Research*, 1953, **6**, 17-S, and personal communication.

exhibits remarkable resistance towards acidic hydrolysis, owing to electrostatic shielding [12] of the polysaccharide chains by the NH_3^+ groups which prevents the approach of hydrions; it was not completely hydrolysed by treatment with 3·3N-hydrochloric acid at 100° for 3 days. After 32 hours' treatment it was converted into a series of chitosaccharides, but because of the low affinity for the absorbent attempts to fractionate the mixture on charcoal–Celite were unsuccessful. However, selective *N*-acetylation [13] of the chitosaccharide mixture followed by chromatography on charcoal–Celite gave the first seven members of a saccharide series of which the first five were obtained crystalline, and the tri- and higher saccharides were hitherto unknown. Table 1 shows the fractions obtained and related data.

Several lines of evidence suggest that the *N*-acetylchitosaccharides are members of a

TABLE 1. *Data on the* N-*acetylated chitosaccharides.*[a]

Fraction [b]	Alcohol (% v/v) in eluate	Saccharide component	No. of monosacch. units	R_{Ac} [c]	Yield [d] (g.)	$[M]_D$
I	2·8—5·0	2-Acetamido-2-deoxy-D-glucose	1	1·00	2·75	+90°
II	10·0—11·6	Di-*N*-acetylchitobiose	2	0·89	1·11	+73
III	15·9—19·1	Tri-*N*-acetylchitotriose	3	0·71	1·20	+14
IV	21·0—23·0	Tetra-*N*-acetylchitotetraose	4	0·60	0·81	−34
V	24·0—26·4	Penta-*N*-acetylchitopentaose	5	0·30	0·70	−94
VI	26·8—28·6	Hexa-*N*-acetylchitohexaose	6	0·10	0·43	−141
VII	29·3—30·1	Hepta-*N*-acetylchitoheptaose	7	0·03	0·09	−181

Fraction [b]	Formula	Found C%	Found H%	Found N%	Found *M* [e]	Required C%	Required H%	Required N%	Required *M*
I	$C_8H_{15}O_6N$	43·6	6·95	6·3	208	43·4	6·8	6·3	221
II	$C_{16}H_{28}O_{11}N_2$	45·1	6·9	6·6	420	45·3	6·6	6·6	424
III	$C_{24}H_{41}O_{16}N_3$	45·7	6·5	6·2	652	45·9	6·5	6·7	627
IV	$C_{32}H_{54}O_{21}N_4$	46·0	6·7	6·8	871	46·3	6·5	6·75	830
V	$C_{40}H_{67}O_{26}N_5$	46·6	6·5	6·05	1050	46·5	6·5	6·8	1033
VI	$C_{48}H_{80}O_{31}N_6$	—	—	—	1225	—	—	—	1236
VII	$C_{56}H_{93}O_{36}N_7$	—	—	—	1317	—	—	—	1439

[a] Obtained by fractionation of a mixture (10·16 g.) on a charcoal–Celite column [7] (420 × 55 mm.) by gradient elution [14] with aqueous ethanol. [b] Intermediate fractions were obtained which contained more than one component. [c] Obtained with pyridine–pentan-2-ol–water as solvent.[15] [d] Total recovery from the column was 86%. [e] Values determined in aqueous solutions.

polymer-homologous series. Thus a plot of $[M]_D/n$ against $(n - 1)/n$, where $[M]_D$ is the molecular rotation and n is the degree of polymerisation (D.P.), yielded a straight line (Fig. 1). Freudenberg *et al.*[16] predicted this result for a homologous series of oligosaccharides when $n > 1$. The change in the sign of $[M]_D$ from positive to negative as n increases indicates β-linkages.

It has been shown [17] that a plot of log $(1/R_F - 1)$ against n, where R_F is the paper-chromatographic mobility and n is the D.P., yields a straight line for a homologous series of oligosaccharides. This relation was observed (Fig. 2) for the R_F values of the *N*-acetylated chitosaccharides obtained with the organic phase of butanol–ethanol–water–ammonia (40 : 49 : 10 : 1). However a plot of the R_F values obtained with pyridine–pentan-2-ol–water [15] (1 : 1 : 1) yielded a smooth curve (Fig. 3), as did the R_F values similarly obtained for the cellosaccharides (Fig. 3). In contrast, a straight-line

[12] Cf. Foster and Huggard, *Adv. Carbohydrate Chem.*, 1955, **10**, 335, and references cited therein.
[13] Roseman and Ludowieg, *J. Amer. Chem. Soc.*, 1954, **76**, 301.
[14] Lindberg and Wickberg, *Acta Chem. Scand.*, 1954, **8**, 569.
[15] Jeanes, Wise, and Dimler, *Analyt. Chem.*, 1951, **23**, 415.
[16] Freudenberg, Friedrich, and Bumann, *Annalen*, 1932, **494**, 41; cf. Whelan, Bailey, and Roberts, *J.*, 1953, 1294; Wolfrom and Dacons, *J. Amer. Chem. Soc.*, 1952, **74**, 5331; Whistler and Chen-Chuan Tu, *ibid.*, p. 3609.
[17] French and Wild, *ibid.*, 1953, **75**, 2612; cf. Martin, *Biochem. Soc. Symp.*, 1949, **3**, 4; Bate-Smith and Westhall, *Biochim. Biophys. Acta*, 1950, **4**, 427; also ref. 15.

plot was obtained with the R_F values obtained for the maltosaccharides (Fig. 3). The similarity in chromatographic behaviour of the *N*-acetylchitosaccharides and the cellosaccharides is striking and the smooth curves obtained indicate the homologous nature of the oligosaccharide series.

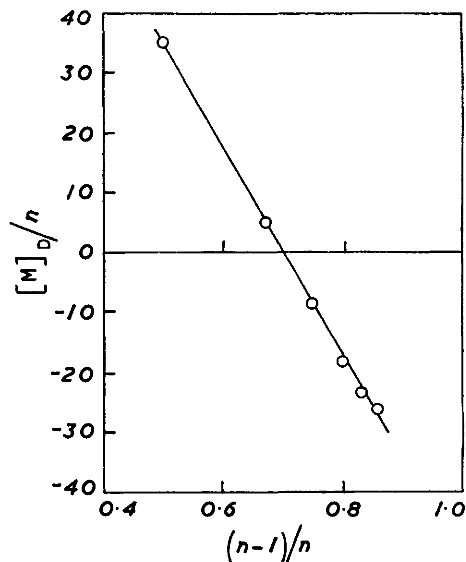


FIG. 1. *Relation between* $[M]_D/n$ *and* $(n-1)/n$ *for* N-*acetylchitosaccharides, where* n = *no. of monosaccharide units.*

FIG. 2.

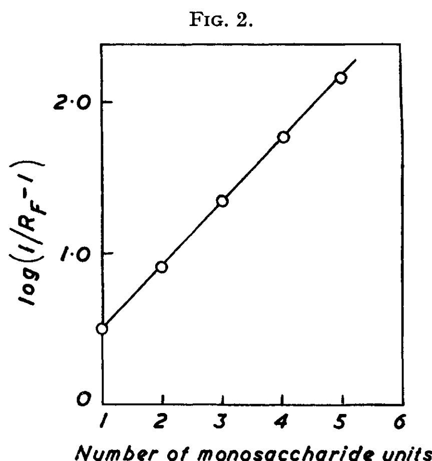
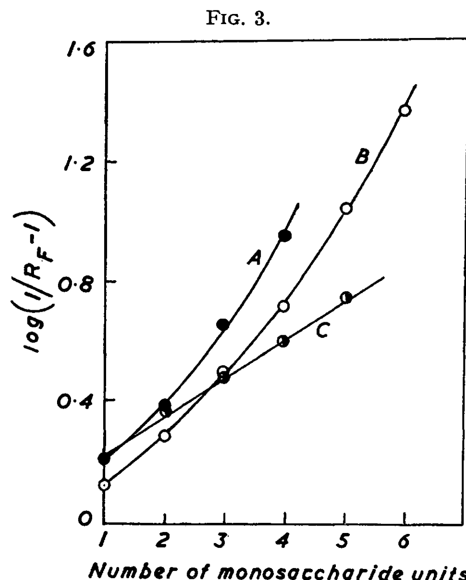


FIG. 3.



Relation between log $(1/R_F - 1)$ *and* n: FIG. 2, N-*acetylchitosaccharides,* R_F *values being determined with the organic phase of butanol–ethanol–water–ammonia* (40 : 10 : 49 : 1); FIG. 3, N-*acetylchitosaccharides* (*A*), *cellosaccharides* (*B*), *and maltosaccharides* (*C*), R_F *values being determined with pyridine–pentan-2-ol–water.*[15]

Infrared-spectral data for the *N*-acetylchitosaccharides and related compounds are shown in Table 2. All the *N*-acetylchitosaccharides and chitin show absorption at 884—900 $cm.^{-1}$ (type 2b) indicative[18] of β-glucopyranosidic linkages, whereas only the

crystalline forms of 2-acetamido-2-deoxy-D-glucose, di-*N*-acetylchitobiose, and tri-*N*-acetylchitotriose showed absorptions at *ca.* 850 cm.$^{-1}$ (type 2a) indicative [18] of α-glucopyranose derivatives. That these saccharides crystallise in the α-form is suggested by the downward mutarotation exhibited in each case on dissolution in water.

TABLE 2. *Infrared absorptions of chitosaccharides and related compounds*

Compound	State [a]	Frequencies [b]					
2-Acetamido-2-deoxy-α-D-glucose [c] ...	X	—	933w	909m	884m	850m	770m
No. of units in N-*acetylchitosaccharides*:							
2	X	966w	947m	905m	892w	840m	—
2	f.d.	961m	947m	—	896m	—	—
3	X	961m	947m	917w	899m, 888m	857w	—
3	f.d.	—	945m	—	892m	—	—
4	f.d.	—	951m	—	892m	—	—
5	f.d.	—	951m	—	894m	—	—
6	f.d.	—	955m	—	900m	850vvw	—
7	f.d.	—	950m	—	900m	860vvw	—
Chitin [d]	—	970m	947m	—	888m	—	—
2-Acetamido-2-deoxy-D-glucitol	X	966m	939m	—	892m, 876s	—	761wb
Di-*N*-acetylchitobi-itol	f.d.	—	942m	—	892m	—	—

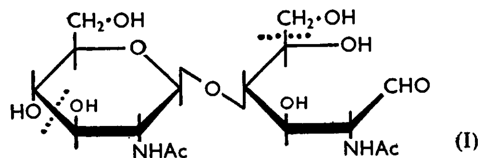
[a] X = crystalline; f.d. = freeze-dried. [b] w = weak; m = medium; s = strong; v = very; b = broad. [c] Data recorded by Barker *et al.*[18] [d] Sample prepared as described by Foster and Hackman.[19]

The general similarity of the infrared spectra of the *N*-acetylchitosaccharides of D.P. >2 was most noticeable and suggested a close structural relation.

The absorption at 892 cm.$^{-1}$ in the spectrum of di-*N*-acetylchitobi-itol cannot be considered diagnostic of a β-linkage since 2-acetamido-2-deoxy-D-glucitol, which contains no glycosidic linkage, absorbs in this range. However, the absence of absorption of type 2a (at *ca.* 850 cm.$^{-1}$) may be regarded as negative evidence in favour of a β-linkage.

The response of acetamido-sugars to the Morgan–Elson test [10] is significantly influenced by substitution of the 3-, 4-, or 6-hydroxyl group.[20] In particular, substitution of the 4-hydroxyl group largely eliminates response to the test by preventing the formation of the furanose form of the amino-sugar derivative.[8] It was observed that whilst di-*N*-acetylchitobiose gave 3—6% of the colour produced by an equimolar quantity of 2-acetamido-2-deoxy-D-glucose the higher *N*-acetylchitosaccharides gave no detectable colour, indicating that the reducing moieties are linked through position 4.

Di-*N*-acetylchitobiose in unbuffered solution at room temperature reduced only 1 mol. of sodium metaperiodate in *ca.* 4 hr. and, with release of 1 mol. of formaldehyde, a further mol. in *ca.* 120 hr. It appears that the non-reducing moiety of the disaccharide is relatively rapidly attacked by periodate (methyl 2-acetamido-2-deoxy-α-D-glucopyranoside consumes 1 mol. in *ca.* 5 hr. under similar conditions). The second mol. of oxidant is



consumed as the *aldehydo*-form of the reducing moiety is attacked (cf. I). The slow reaction indicates the presence of a small proportion of the *aldehydo*-form in equilibrium with the pyranose form (the furanose form is precluded), which accords with the general theory of the composition of the equilibria of the reducing forms of sugars in aqueous

[18] Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171.
[19] Foster and Hackman, *Nature*, 1957, **180**, 40.
[20] Jeanloz and Trémège, *Fed. Proc.*, 1956, **15**, 282.

solution.[21] Similar results were observed with tri-*N*-acetylchitotriose. Slow overoxidation occurred with both di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose.

Sodium borohydride readily reduced di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose to, respectively, di-*N*-acetylchitobi-itol and tri-*N*-acetylchitotri-itol. The former alcohol rapidly (<10 min.) consumed *ca.* 1 mol. of periodate and released *ca.* 1 mol. of formaldehyde; a second mol. was consumed relatively slowly (*ca.* 11 hr.). Rapid attack at the acyclic moiety would be expected from both known data [22] and the observed rapid reaction of 2-acetamido-2-deoxy-D-glucitol with periodate. Similarly tri-*N*-acetylchito-tri-itol rapidly (<10 min.) consumed *ca.* 1 mol. of periodate and released *ca.* 1 mol. of formaldehyde, and slowly (*ca.* 24 hr.) consumed a second mol. of oxidant; acidic hydrolysis of the product formed on complete oxidation released 2-amino-2-deoxy-D-glucose, presumably from the central moiety of the molecule.

The optical rotations, infrared spectra, periodate oxidation patterns, and colorimetric tests described for di-*N*-acetylchitobiose and its reduced derivative substantiate the $\beta 1 \longrightarrow 4$ linkage previously allocated to the disaccharide on the basis of other evidence.[6,23]

Experimental

Isolation of Chitin.—Cleaned and fragmented shells of the common edible crab, *Cancer pagurus*, were twice digested overnight at room temperature with excess of 2N-hydrochloric acid. After being washed free from acid the shells were dried at 100° and then ground in a ball-mill (steel balls) until most of the product passed through a 60-mesh sieve.

Crude powdered chitin (490 g.) was suspended in 2N-hydrochloric acid for 48 hr., then collected by centrifugation, washed with water, and extracted with aqueous sodium hydroxide (2 l.; 5% w/v) for 12 hr. at 100°. The product was isolated from the hot suspension by centrifugation and the alkaline extraction repeated four times. The final residue was freed from alkali by washing with water, neutralisation with hydrochloric acid, and prolonged dialysis against water. After drying, the chitin (277 g.) was obtained as a straw-coloured powder [Found: C, 44·6; H, 6·4; N, 6·2. Calc. for $(C_8H_{13}O_5N)_n$: C, 47·3; H, 6·4; N, 6·9%].

Acetolyses of Chitin.—(*a*) Chitin (10 g.) was added to a cooled mixture of acetic anhydride (50 ml.) and concentrated sulphuric acid (6·5 ml.). After storage at room temperature for 40 hr. and then at 55° for 11 hr. the mixture was added to a cooled (0°) solution of sodium acetate trihydrate (40 g.) in water (260 ml.). Insoluble material was removed by centrifugation and, after several hours, the supernatant liquid was neutralised with solid sodium carbonate and extracted with chloroform (3 × 200 ml.). The combined extracts were washed with water, dried (Na_2SO_4), and concentrated, and the residue was triturated with methanol. Recrystallisation of the crude product (3 g.) from methanol gave chitobiose octa-actate as colourless needles (1·14 g.), m. p. 286—288°. A second recrystallisation gave the product (0·2 g.), m. p. 308—309° (decomp.), $[M]_D^{18}$ +372° (*c* 0·5 in acetic acid) (Found: C, 49·4; H, 5·9; N, 4·3. Calc. for $C_{28}H_{40}O_{17}N_2$: C, 49·7; H, 5·9; N, 4·1%).

(*b*) Chitin (125 g.) was acetolysed in several portions as described in (*a*) and, after neutralisation, the insoluble material was extracted with chloroform and the extract added to the chloroform extract of the supernatant liquor. The combined extracts were washed with water, dried (Na_2SO_4), and concentrated and the residue was triturated with methanol to yield, after drying *in vacuo* (P_2O_5), a mixture (17·46 g.) of acetylated chitosaccharides.

A solution of this material (16·1 g.) in dry methanol (700 ml.) was deacetylated by the addition of a trace of metallic sodium [α_D (2 dm. tube) +0·09° $\longrightarrow$ +0·65° (constant)]. After 40 hr. the solution was diluted with water, freed from cations by Zeo-Carb 225 (H^+ form), and evaporated under diminished pressure, to yield the product (9·9 g.). Paper chromatography with the organic phase of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1) and detection with aniline hydrogen phthalate [24] and alkaline silver nitrate [25] revealed five components with R_{Ac} values (mobility relative to that of 2-acetamido-2-deoxy-D-glucose) 0·24, 0·52, 0·78, 1·00, and 1·87.

[21] See Pigman, " The Carbohydrates," Academic Press, New York, 1957, p. 54.
[22] Bobbitt, *Adv. Carbohydrate Chem.*, 1956, **11**, 1.
[23] Zechmeister and Tóth, *Enzymologia*, 1939, **7**, 165.
[24] Partridge, *Nature*, 1949, **164**, 443.
[25] Trevelyan, Proctor, and Harrison, *ibid.*, 1950, **166**, 444.

A solution of the mixed products (10·4 g.) in water (100 ml.) was introduced on to a charcoal–Celite column [7] (400 × 57 mm.), and eluted successively with water (4 l.) and aqueous ethanol of ethanol concentrations (v/v) 2·5% (5 l.), 5% (14 l.), 7·5% (15·5 l.), 10% (13 l.), and 15% (5·5 l.); 86% of the original mixture was thus eluted. In this and similar column separations up to eleven products, R_{Ac} 0·11—2·73, could be detected on analysis of the 50 ml. fractions collected. The principal fractions are recorded in Table 3

TABLE 3.

Fraction	Vol. of eluate (l.)	Main component (R_{Ac} value)	Yield (g.)
I	3—7·5	2-Acetamido-2-deoxy-D-glucose (1·00)	0·48
II	10·5—24·5	Di-*N*-acetylchitobiose (0·52)	5·54
III	42·5—49·5	Tri-*N*-acetylchitotriose (0·24)	1·22

A solution of fraction II (5·2 g.) in water (50 ml.) was introduced on to a charcoal–Celite column [7] (370 × 57 mm.) and the column subjected to gradient elution [14] with aqueous alcohol; the alcohol concentration was increased at the rate of 1·67% per litre. The di-*N*-acetylchitobiose emerged from the column, together with other components, in the eluate range 8·75—11·4 l. Concentration of the fraction 10·15—10·6 l. containing di-*N*-acetylchitobiose together with traces of two other components gave a product (2·69 g.) which, after crystallisation from aqueous methanol, gave the biose [2-acetamido 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose] as colourless needles (1·71 g.), m. p. 260—262° (decomp.), $[\alpha]_D$ +25·2° (3 min.) ⟶ +17·2° (equilibrium), $[M]_D$ +73° (*c* 0·5 in H_2O). The disaccharide contained *ca.* 0·23% of incombustible material. The analytical data are recorded in Table 1.

Recrystallisation of fraction III (0·4 g.) from aqueous methanol gave tri-*N*-acetylchitotriose (0·2 g.) as microscopic, colourless needles, m. p. 304—306° (decomp.), $[\alpha]_D$ +3·8 (13 min.) ⟶ +2·2° (equil.), $[M]_D$ +14° (*c* 0·9 in H_2O).

In a separate experiment a crude mixture (24 g.) of deacetylated products, obtained as in (*b*), was dissolved in water (250 ml.) and introduced on a charcoal–Celite column [7] (210 × 50 mm.). Elution was initially with water (3 l.) and later with aqueous ethanol, the concentration of ethanol increasing at the rate of 5% per l. for 6 l. and at 10% per l. for the next 3 l. Concentration of the eluate fraction 5·1—5·25 l. gave a product (0·18 g.) which contained several components. Crystallisation from dry methanol gave *compound A* (37 mg.), m. p. 175—177° (Found: C, 46·8; H, 6·4; N, 6·8. $C_8H_{13}O_5N$ requires C, 47·3; H, 6·4; N, 6·9%). This reacted strongly, on paper chromatograms, with aniline hydrogen phthalate, and with the organic phase of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1) had R_{Ac} 1·87.

*De-*N*-acetylation of Chitin.*—Chitin (21 g.; H_2O content 6·7%) and potassium hydroxide (40 g.) were heated together at 170—190° in a current of nitrogen for 95 min. The cooled mixture was treated with water (250 ml.) and concentrated hydrochloric acid (64 ml.). After dialysis of the product against running tap water for 42 hr. and against distilled water for 5 hr., insoluble material (3 g.) was removed by centrifugation and the supernatant liquid concentrated by freeze-drying, to yield chitosan hydrochloride (11·4 g.) [Found: Cl, 13·1. Calc. for $(C_6H_{12}O_4NCl)_n$: Cl, 17·95%]. The chloride content of the chitosan hydrochloride could not be determined by direct precipitation of silver chloride since the salt failed to coagulate and precipitate in the presence of the polysaccharide. The following procedure proved to be satisfactory: Chitosan hydrochloride (0·4 g.) was suspended in 0·225N-sulphuric acid (37 ml.) and treated with 2·76% w/v aqueous sodium nitrite (37 ml.) at room temperature for 45 min. Silver chloride was then precipitated and determined by the usual method. A control experiment was performed on 2-amino-2-deoxy-D-glucose hydrochloride (Found: Cl, 16·3. Calc. for $C_6H_{14}O_5NCl$: Cl, 16·5%).

Acidic Hydrolysis of Chitosan Hydrochloride.—The following conditions appeared to be optimum for the production of a series of oligosaccharides. A solution of chitosan hydrochloride (3·1 g.) in water (210 ml.) was heated to 100° and concentrated hydrochloric acid (90 ml.) added. The solution was kept at 100° for 34 hr., then cooled, decolorised with activated charcoal, and neutralised with methyldi-*n*-octylamine.[26] Paper chromatography of the hydrolysate with pyridine–pentan-2-ol–water (1 : 1 : 1) as solvent [15] and detection with aniline hydrogen phthalate [24] or ninhydrin revealed the presence of mono- to hexa-saccharide. Attempts to fractionate the mixture on charcoal–Celite [7] failed because it was too weakly absorbed and was eluted by water.

[26] Smith and Page, *J. Soc. Chem. Ind.*, 1948, **67**, 48.

A solution of the saccharide mixture (2·97 g.) in water (75 ml.) was treated at 0° with methanol (7·5 ml.), Amberlite IRA-400 (CO_3^{2-} form; 90 ml.) and acetic anhydride (2 ml.); the stirred mixture was stored at 0—5° for 1·5 hr. Thereafter the resin was removed and replaced by Amberlite IR-120 (H^+ form, 4 ml.) in order to remove any free amino-sugars. The de-ionised solution was concentrated to 50 ml., insoluble material (0·46 g.) was removed by centrifugation, and the supernatant liquid concentrated by freeze-drying to yield a mixture (1·96 g.) of *N*-acetylchitosaccharides. Paper chromatography of the *N*-acetylchitosaccharide mixture with pyridine–pentan-2-ol–water [15] and detection with alkaline silver nitrate [25] revealed a series of at least six components with R_{Ac} <1·0 and a small amount of a component of R_{Ac} 1·2.

A similar *N*-acetylchitosaccharide mixture (10·16 g.), dissolved in water (110 ml.), was introduced on to a charcoal–Celite column [7] (420 × 55 mm.) and submitted to gradient elution with aqueous ethanol; the ethanol concentration increased at the rate of 1·67% per l. The fractions which contained pure components are recorded in Table 1 together with other data.

Crystallisation of fractions III (0·9 g.), IV (0·69 g.), and V (0·68 g.) from aqueous methanol gave respectively: tri-*N*-acetylchitotriose (0·63 g.), m. p. 309—311° (decomp.), $[\alpha]_D^{17}$ +2·5° ± 0·4° (equil.; *c* 1·2 in H_2O), $[M]_D$ +16°, *tetra*-N-*acetylchitotetraose* (0·39 g.), m. p. 290—300° (decomp.), $[\alpha]_D^{20}$ −4·1° ± 0·2°, (equil.; *c* 1·0 in H_2O), $[M]_D$ −34°, and *penta*-N-*acetylchitopentaose* (0·3 g.), m. p. 285—295° (decomp.), $[\alpha]_D^{20}$ −9·1° ± 0·2° (equil.; *c* 1·0 in H_2O), $[M]_D$ −94°, all small colourless needles.

All the *N*-acetylchitosaccharides, before crystallisation, contained significant amounts of incombustible material which was considerably reduced but not eliminated after crystallisation. Hexa-*N*-acetylchitohexaose, $[\alpha]_D$ −11·4° ± 0·4° (equil.; *c* 0·8 in H_2O), $[M]_D$ −141°, and hepta-*N*-acetylchitoheptaose, $[\alpha]_D$ −12·6° ± 1·9° (equil.; *c* 0·3 in H_2O), $[M]_D$ −181°, were not obtained crystalline; both appeared homogeneous on paper chromatography. Analytical and other data for these saccharides are recorded in Table 1.

Acetylation of Tri-N-*acetylchitotriose*.—The trisaccharide (0·25 g.) was treated with acetic anhydride (2·5 ml.) and pyridine (2·5 ml.) at room temperature for 2 days. Insoluble material was collected by centrifugation and re-treated with the reagents. The combined reaction mixtures were freed from insoluble material by centrifugation and diluted with ice-water (125 ml.). The solution was extracted with chloroform (3 × 30 ml.), the combined extracts were washed successively with water, aqueous sodium hydrogen carbonate, and water, then dried (Na_2SO_4), and concentrated. Recrystallisation of the residue gave *chitotriose hendeca-acetate* (50 mg.) as colourless needles, m. p. 304—305° (decomp.), $[\alpha]_D^{18}$ +30° ± 2° (*c* 0·5 in acetic acid) (Found: C, 49·6; H, 5·8; N, 4·3. $C_{40}H_{57}O_{24}N_3$ requires C, 49·8; H, 5·9; N, 4·4%).

Hypoiodite Oxidation of the N-*Acetylated Chitosaccharides*.[27]—Preliminary experiments revealed that overoxidation of 2-acetamido-2-deoxy-D-glucose occurred if the reaction was performed at room temperature. All oxidations were therefore carried out at 0°.

Samples of the *N*-acetylchitosaccharides in Table 2 (1·5, 1·0, and 0·67 mg.) were separately dissolved in water (*ca.* 0·5 ml.) and treated successively with a mixture (1 ml. containing equal parts by volume of 0·2M-sodium carbonate and 0·2M-sodium hydrogen carbonate) and 0·02N-iodine (1 ml.). The reaction solutions were stored at 0—2° for 18 hr., then acidified with 5N-sulphuric acid (10 ml.), diluted with water (*ca.* 10 ml.), and titrated with standard thio-sulphate solution. The results (average of three determinations) are recorded in Table 1.

Behaviour of the N-*Acetylated Chitosaccharides in the Morgan–Elson Test*.—Samples (1 ml.) of aqueous solutions (containing *ca.* 100 μg./ml.) of the saccharides in Table 2 and 2-acetamido-2-deoxy-D-glucose (containing 100, 70, and 40 μg./ml.) were treated with sodium carbonate and *p*-dimethylaminobenzaldehyde as described by Aminoff, Morgan, and Watkins.[10] The colour which developed was measured with a Spekker photoelectric absorptiometer with Ilford No. 605 filters.

No detectable colour was produced by tri-*N*-acetylchitotriose and the higher saccharides, but di-*N*-acetylchitobiose gave *ca.* 3—6% of the colour produced by an equimolar amount of the monosaccharide.

Reduction of Di-N-*acetylchitobiose*.—A solution of this biose (0·4 g.) in water (12 ml.) was treated with sodium borohydride (31 mg.) in water (4 ml.) at room temperature for 2·5 hr. (In preliminary experiments the reaction was followed polarimetrically and shown to be essentially complete within 60 min.) Dilute acetic acid was then added dropwise until excess

[27] Cf. Jeanloz and Forchielli, *Helv. Chim. Acta*, 1950, **33**, 1690.

of borohydride was decomposed, and the solution was de-ionised by Amberlite resins IR-120 (H^+ form; 4 ml.) and IRA-400 (HO^- form; 4 ml.). Concentration of the solution gave a solid residue (0·38 g.) which appeared homogeneous on paper chromatography. Recrystallisation from ethanol gave *di*-N-*acetylchitobi-itol* [*2-acetamido-*4-O-(*2-acetamido-2-deoxy-*β-D-*glucopyranosyl*)-*2-deoxy-*D-*glucitol*] *monohydrate* as extremely hygroscopic large colourless plates (0·22 g.), m. p. 102—105°, $[M]_D$ −51° (*c* 1·6 in H_2O) (Found: C, 43·2; H, 7·1; N, 6·4. $C_{16}H_{30}O_{11}N_2,H_2O$ requires C, 43·2; H, 7·2; N, 6·3%). A sample, dried to constant weight *in vacuo* at 60° over phosphoric oxide, lost 4% of its weight when the temperature was raised to 130° (Calc.: H_2O, 4·05%).

Reduction of Tri-N-*acetylchitotriose*.—The trisaccharide (0·4 g.) was reduced as for the disaccharide, to yield a product (0·35 g.) which on crystallisation from ethanol gave *tri*-N-*acetylchitotri-itol* as very hygroscopic colourless needles (0·15 g.), m. p. 195—200°, $[M]_D$ −64° (*c* 1·0 in H_2O) (Found: C, 45·5; H, 7·0; N, 6·5. $C_{24}H_{43}O_{16}N_3$ requires C, 45·8; H, 6·8; N, 6·7%)

Periodate Oxidations.—The following general procedure was adopted. A solution of the substrate (*ca.* 0·13 mmole) in water (*ca.* 25 ml.) was treated with 0·25M-sodium metaperiodate (15 or 5 ml.), and the volume rapidly adjusted to 50 ml. The consumption of periodate was determined on aliquot parts by the standard arsenite method. The blue colour produced at the end-point when using Thyodene (starch–urea mixture) as indicator, faded rapidly in the analysis of samples withdrawn after *ca.* 2 hr. The effect, which presumably is due to some form of overoxidation, could be minimised by performing titrations at $<10°$.

Acidity which developed in the solutions was determined in aliquot parts, after destruction of excess of periodate with ethylene glycol, by titration with standard alkali. Formaldehyde was determined by the chromotropic acid method.[28]

The following results were obtained at room temperature (12—18°):

(*a*) 2-Acetamido-2-deoxy-D-glucose (0·005106M) in sodium metaperiodate (0·075M):

Time (hr.)	0·1	0·25	0·5	1·0	3·0	6·0	21
Oxidant consumption (mol.)	2·11	2·90	3·66	4·55	4·88	4·96	5·23

After 25 hr. acidity corresponding to 2·16 mols. of formic acid was developed.

(*b*) Di-*N*-acetylchitobiose (0·002604M) in sodium metaperiodate (0·075M):

Time (hr.)	1	4	23	44	92	140	188	260
Oxidant consumption (mol.)	—	1·04	1·55	1·78	1·93	2·07	2·16	2·37
Formaldehyde liberation (mol.)	0·09	0·18	0·55	0·72	0·91	0·97	0·97	—

After 140 hr. acidity corresponding to 0·08 mol. of formic acid had developed.

(*c*) Tri-*N*-acetylchitotriose (0·002632M) in sodium metaperiodate (0·075M):

Time (hr.)	3	24	50	72	120	168
Oxidant consumption (mol.)	1·05	1·81	2·15	2·19	2·24	2·38
Formaldehyde liberation (mol.)	0·20	0·77	0·96	1·02	1·03	1·03

Development of acidity (as mol. of formic acid) in 72 hr. was 0·08 mol., and in 216 hr. 0·30 mol.

(*d*) 2-Acetamido-2-deoxy-D-glucitol (0·002664M) in sodium metaperiodate (0·025M):

Time (hr.)	0·25	1·0	20
Oxidant consumption (mol.)	2·96	3·00	3·00
Formaldehyde	1·01	1·01	1·01

(*e*) Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (0·002596M) in sodium metaperiodate (0·025M):

Time (hr.)	0·2	0·5	1·0	2·0	4·25	21	30
Oxidant consumption (mol.)	0·19	0·34	0·49	0·68	0·89	1·02	1·02

(*f*) Di-*N*-acetylchitobi-itol (0·002624M) in sodium metaperiodate (0·025M):

Time (hr.)	0·16	1·0	3·0	5·75	11·0	24	48
Oxidant consumption (mol.)	0·98	1·13	1·40	1·69	1·92	2·07	2·07
Formaldehyde liberation (mol.)	1·07	0·99	1·02	—	—	1·02	—

No formic acid was produced in 48 hr.

(*g*) Tri-*N*-acetylchitotri-itol (0·002652M) in sodium metaperiodate (0·025M):

Time (hr.)	0·16	1·0	3·0	6·0	10·0	24	48
Oxidant consumption (mol.)	0·89	1·04	1·28	1·64	1·79	1·90	1·95
Formaldehyde liberation (mol.)	0·96	0·98	0·98	—	—	0·97	—

No formic acid had been produced in 48 hr.

[28] O'Dea and Gibbons, *Biochem. J.*, 1953, **55**, 580.

The reaction solution (*g*) (5 ml.) after 48 hours was treated with excess of ethylene glycol (0·1 ml.) and, after a few minutes, with a solution of sodium borohydride (5 mg.) in water (1 ml.) at room temperature for 1 hr. After decomposition of the reducing agent with acetic acid the solution was de-ionised with Amberlite resins IR-120 (H^+ form) and IRA-400 (HO^- form) and concentrated to 2 ml. The solution was made 2N with respect to hydrochloric acid, heated at 100° for 2 hr., neutralised with methyldi-*n*-octylamine and subjected to ionophoresis (neutral buffer). A component was present with behaviour identical with that of 2-amino-2-deoxy-D-glucose.

Infrared Spectra.—Infrared spectra were measured by the potassium bromide disc method with a Grubb-Parsons single-beam spectrometer.

The authors thank Dr. D. H. Whiffen for advice on the infrared spectra. One of them (J. M. W.) thanks the D.S.I.R. for a maintenance award.

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[*Received, December 30th,* 1957.]