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INTERACTIONS OF CONCANAVALIN A WITH α-D-GLUCANS

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ABSTRACT

The interaction of α-D-glucans with Concanavalin A has been studied by turbidimetry. Amylopectins from various botanical sources and two glycogens were characterised for their fine structure [% (1→6)-α- linkages, % beta-amylolysis, iodine interaction] and their molecular size (intrinsic viscosity, gel permeation). Branched α-D-glucans with defined structures were prepared either by enzymic (beta-amylolysis or phosphorolysis of oyster glycogen and amylopectins) or mild acid hydrolysis of waxy-maize amylopectin. Selection of high concentrations of Con A (>0.9 mg/mL) and (20mM, Tris-HCl) buffer allow high sensitivity for studying the interactions. Two structural features of branched polysaccharides are demonstrated to be major parameters for the interaction, namely, the hydrodynamic volume and the external chain-length. At pH 7.0, the Con A interaction modifies the beta-amylolysis kinetics by decreasing the affinity constant of beta-amylase, whereas no modification was detected at pH 5.2.

INTRODUCTION

Concanavalin (Con A) from *Canavalia ensiformis* is a lectin which binds the non-reducing terminal α-D-glucopyranosyl or α-D-mannopyranosyl groups of any molecule or particle, to form insoluble complexes[1,2]. Neutral polysaccharides, such as α-D-glucans, mannans, and dextrans, interact[3] with Con A, whereas such polyelectrolytes as heparin, D-glucan phosphates, galactan sulfate, fucan, RNA, and bacterial lipopolysaccharides[4,5] interact but with no chemical specificity. The polysaccharide interactions are differentiated on the basis of their "glycogen-value" (g.v.), which is the turbidity ratio obtained by comparing a given polysaccharide and the same quantity of rabbit-liver glycogen in a standard assay[4]. This molecular interaction depends on the lectin concentration, the ligand concentration, and the reaction time[6,7].

The main rule for selectivity is based on the need for a branched structure to support the terminal ligands. However, the primary chemical structure of the interacting macromolecules is generally very complex and known only through chemical methods which reflect an average structure. Therefore, the molecular parameters

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governing this multivalent system involving macromolecules are still not fully understood[3].

α-D-Glucans, either linear (amylose, pullulan) or branched (glycogen[8], amylopectin[9]), are ideal macromolecules for such studies, as their structures are now well established[10,11] and are easily modified by enzymic[12] and chemical means. Thus, beta-amylase and phosphorylase, as exo-enzymes, depolymerise the outer chains of these macromolecules, starting from the non-reducing chain ends, until blocked by branch points. The resulting limit-dextrins differ in the lengths of the outer chains (maltosyl or maltotriosyl stubs for the products of beta-amylase action and maltotetraosyl stubs for those of phosphorylase action). In contrast, acid-catalysed depolymerisation occurs in a random manner[13].

The reactivity of glycogen towards Con A was recognised[4,14–16] first, whereas amylopectin interacts with Con A to a lesser extent and only in the presence of higher concentrations of lectin[2,6]. By using enzymically modified polysaccharides, Smith *et al.*[6] demonstrated that the number of chain ends by unit weight is the major parameter. However, such variables as hydrodynamic volume, polydispersity, and external chain-length were not studied systematically.

We now report on the molecular parameters which affect the formation of complexes between Con A and amylopectins, modified by enzymes (beta-amylase or phosphorylase) or chemically (acid), and on the effects of beta-amylase on Con A–amylopectin complexes.

MATERIALS AND METHODS

Materials. — Highly purified Con A (Grade V) (Sigma), phosphorylase (EC 1.1.3.4) from rabbit muscle (Boehringer), isoamylase (EC 3.2.1.68) of *P. amyloderamosa* (Hayashibara), sweet-potato beta-amylase (EC 3.2.1.2) (Koch–Light), *A. niger* amyloglucosidase (EC 3.2.1.3) (Merck), *A. niger* D-glucose oxidase (EC 1.1.3.4) (Grade II) and horse-radish peroxidase (EC 1.11.1.7) (Grade I) (Sigma) and 2,2′-azino-di(3-ethylbenzothiazoline sulphonic acid) (Boehringer) were used. Cross-linked agarose (Sepharose Cl-2B, Pharmacia) and hydrophilic vinyl polymer HW-75 (Merck) were used for gel-permeation chromatography.

Oyster, rabbit-liver, and *Crepidula fornicata* glycogens (Sigma), and potato amylose (Avebe) were used without further purification. Amylopectins from faba bean, normal and waxy maize, *Manihot*, potato, rice, smooth pea, and wheat were obtained from native starch after selective removal of amylose by complexation with thymol[17].

Beta-limit dextrins corresponding to the remaining part of *Manihot*, waxy-maize, smooth-pea, and wheat amylopectins and oyster glycogen, after treatment with beta-amylase, were prepared according to the procedure of Mercier[18]; ϕ-dextrins from smooth-pea amylopectin and oyster glycogen, after the action of rabbit-muscle phosphorylase, were prepared according to the procedure of Walker and Whelan[19].

Acid hydrolysis. — Amylopectin from waxy-maize starch (0.4%) was dissolved by autoclaving in distilled water for 1 h at 37 p.s.i. (140°). Then hydrolysis was carried out in 2M HCl at 25°. Aliquots were removed at intervals, neutralised with M NaOH, dialysed against distilled water (72 h), and then precipitated with ethanol and acetone.

All other reagents were of analytical grade.

Determination of average chain-length. — The average chain-lengths ($\overline{c.l.}$) of polysaccharides were determined after debranching with isoamylase[20].

Gel-permeation chromatography. — Sepharose CL-2B in 0.1M KOH was used as described by Colonna and Mercier[21]. For HW-75, a column (2.2 × 90 ±1 cm) was eluted with an upward flow of degassed 0.1M KOH at 10 mL/h at room temperature. The concentration of polysaccharide in each fraction (2–3 mL) was determined by the orcinol–sulfuric acid procedure[22]; the λ_{max} of its iodine complex was measured as previously described[21].

Turbidity reaction. — Each reaction was initiated by the rapid addition of 0.1% Con A solution (1.8 mL) to polysaccharide solution (0.2 mL), prepared as follows. Polysaccharide (10–30 mg) was first dissolved in 2.25M NaOH (10 mL). After complete dissolution (at least 30 min), this solution was neutralised with M HCl and 50mM Tris-HCl buffer (40 mL, pH 7.2) containing 0.15M NaCl, mM $CaCl_2$, mM $MgCl_2$, and mM $MnCl_2$. The volume was then adjusted to 100 mL. At least five solutions are obtained by dilution of the mother polysaccharide solution. Con A solution was prepared by dissolving 1 mg of Con A per mL of 20mM tris-HCl buffer (pH 7.2) containing 60mM NaCl, 0.4mM $CaCl_2$, 0.4mM $MgCl_2$, and 0.4mM $MnCl_2$.

The development of the turbidity was monitored spectrophotometrically, using a Beckman 25 instrument at 420 nm, and recorded as absorbance (A) units. The slope of the linear regression between A and concentration of polysaccharide was computed in the range where a linear relationship was observed for each polysaccharide. The corresponding glycogen value (g.v.) was obtained by dividing the slope with similar ratio of rabbit-liver glycogen (g.v. = 1).

Protein concentration. — This was determined by the method of Lowry *et al.*[23] with bovine serum albumin as the standard, and the concentration of polysaccharide by the dual enzymic amyloglucosidase–D-glucose oxidase method[24].

Beta-amylolysis. — The initial rates for waxy-maize amylopectin in the presence of Con A were studied. Eight (10 mL, 0.05–1 mg/mL) solutions of polysaccharide in 50mM citrate–phosphate buffer (pH 7.0 or 5.2) were hydrolysed by 8.4 nkat of beta-amylase at 30°; the initial rate was studied during the first 90 s by measuring the increase in the concentration of maltose (reducing power determined by the Nelson method). The complete reaction was followed by the same technique, at three concentrations (0, 100, and 300 μg/mL) of Con A in two different buffers (50mM citrate–phosphate, pH 5.2 and 7.0), on a solution of waxy-maize amylopectin (800 μg/mL) with 8.4 nkat of beta-amylase in 10 mL of solution.

Iodine staining[25]. — To a solution (0.5 mL) of polysaccharide (5 mg/mL) were added water (9 mL) and a solution (0.5 mL) containing 0.2% of I_2 and 2% of

TABLE I

CHARACTERISTICS OF NATIVE α-D-GLUCANS

						Gel-permeation chromatography			
	I b.c.[a]	λ_{max} *(nm)*	*Beta-amylolysis (%)*	$\overline{C.I.}$	*Intrinsic viscosity (mL/g)*	*Phase*	*Excluded material (%)*	K_{av} *of main peak*	*G v.*
Amylopectin									
Waxy maize	0.1	535	55	20	114	HW-75	86.5	0	5 8
Normal maize	0.9	540	54	20	116		11.2	0.18	5 9
Wheat	0 2	540	55	19.5	125		30.0	0.08	5.8
Rice	0.5	540	57	N.d.[b]	145		N d.	N.d.	3.2
Smooth-pea	1.1	545	57	25	126		17.1	0 16	4.1
Faba bean	0.9	550	56	N.d	155		31 9	0.03	3.8
Manihot	0 8	535	57	22	159		9.5	0 29	6 1
Potato	0 6	545	56	N.d.	164		7.9	0 25	6.3
Glycogen									
Oyster	0.2	480	37	12	2	CL-2B	1 4	0.60	1.3
Crepidula	0.1	490	31	12	5		5 9	0 56	1.1

[a]Expressed as mg of iodine bound per 100 mg of polysaccharide. [b]Not determined.

KI. After staining for 30 min, the absorbance was measured in the range 600–400 nm. All the absorption peaks were broad and covered 20–30 nm. The λ_{max} values reported represent the mid-points and are significant only to ±15 nm.

All other determinations were performed as described previously[21,24,26,27].

RESULTS

Macromolecular characteristics of polysaccharides. — All the polysaccharides studied are composed mainly of α-D-glucose, as proved by amyloglucosidase action (glucose recovery >99%), and their characteristics are compiled in Table I. The λ_{max} values of the iodine complexes of the amylopectins are in the range 535–550 nm, in contrast to those (480–490 nm) for the two glycogens. Nevertheless, the iodine-binding capacities (i.b.c.) were always lower than 1.1 mg of iodine bound per 100 mg of polysaccharide. Beta-amylolysis limits of the amylopectins were 55–57%, whereas those of glycogens were 31–37% (Table II). When treated with phosphorylase, smooth-pea amylopectin was 44% transformed into D-glucose 1-phosphate, in contrast to a value of 33% for the oyster glycogen. The $\overline{c.l.}$ values for the glycogens and the amylopectins, computed by the relation $\overline{c.l.} = 100/[\%(1\rightarrow6)\text{-}\alpha$ linkages], are estimated to be 19–25 and 12, respectively. External chain-length ($\overline{e.c.l.}$) was calculated from the beta-amylolysis limit, according to the relation:

$$\overline{e.c.l.} = \left[\frac{\text{beta-amylolysis limit (\%)}}{100} \times \overline{c.l.}\right] + 2.$$

The $\overline{e.c.l.}$ values obtained for the amylopectins and glycogens were 12.5–13.5 and 7, respectively. Intrinsic viscosities of native amylopectins were higher (114–164 mL/g) than those (~2–5 mL/g) of glycogens, which were difficult to determine precisely due to the low specific viscosity of their solutions.

TABLE II

ENZYMIC MODIFICATIONS OF AMYLOPECTINS AND OYSTER GLYCOGEN

	G.v.[a]	*Phosphorolysis*		*Beta-amylolysis*	
	Native polysaccharide	*Phosphorolysis (%)*	*G.v.*	*Hydrolysis (%)*	*G.v.*
Glycogen, oyster	1.3	33	1.2	37	1.0
Amylopectin					
Waxy maize	5.8	N.d.[b]	N.d.	55	4.5
Wheat	5.8	N.d.	N.d.	55	4.6
Manihot	6.1	N.d.	N.d.	57	4.1
Smooth pea	4.1	44	3.7	57	3.4

[a]Expressed on an equal weight basis. [b]Not determined

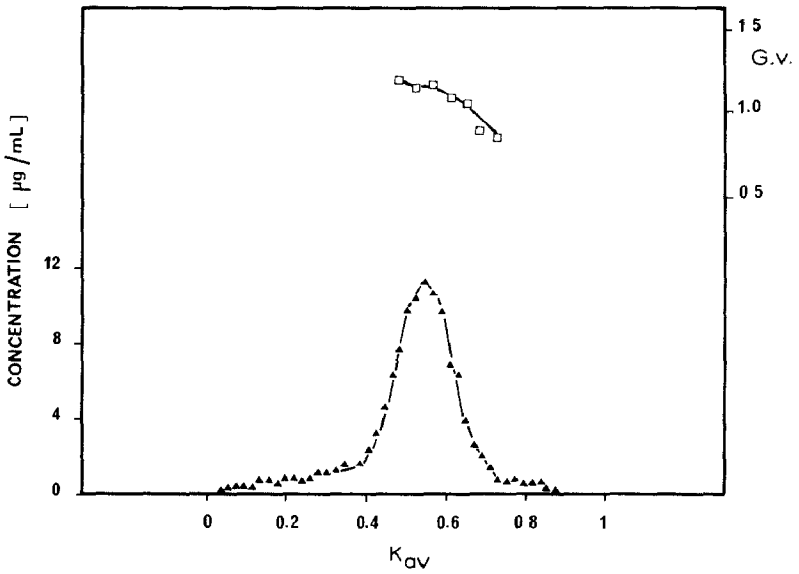

Fig. 1. Elution profile on Sepharose CL-2B of oyster glycogen (—▲—) and the corresponding g.v (—□—).

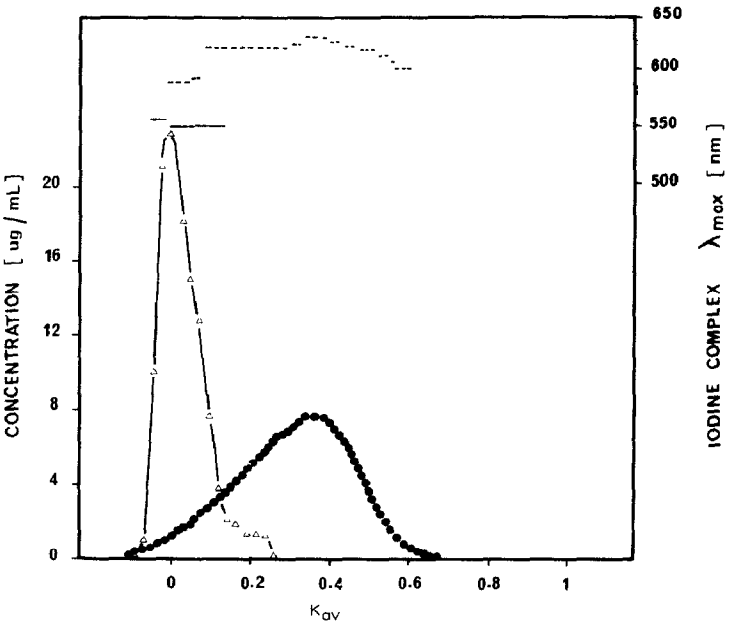

Fig. 2. Elution profiles on Sepharose CL-2B of potato amylose (—●—) and smooth-pea amylopectin (—△—).

When chromatographed on Sepharose CL-2B, all the glycogens studied gave one peak in the middle (K_{av} 0.56–0.60) of the fractionation range (Fig. 1). Their low iodine-binding capacities hindered the determination of the λ_{max} of the iodine–polysaccharide complex for each fraction.

The profile of potato amylose on the same column contained one broad peak located at K_{av} 0.37, with λ_{max} increasing from 595 (K_{av} 0.0) to 630 nm (K_{av} 0.19–0.67) (Fig. 2). In contrast, all the amylopectins studied (Fig. 2) showed sharp peaks at the void volume, representing the main part (70.0–97.2%) of these molecules. The λ_{max} of the iodine complexes of these fractions was always 540 nm. The tails, following the excluded material, were restricted to K_{av} <0.62 (waxy maize), with a λ_{max}, when the polysaccharide concentration was >10 μg/mL, in the same range 535–550 nm. However, HW-75 was a gel suitable for the molecular sieving (Fig. 3) of these branched macromolecules, which were eluted as one single and broad peak (K_{av} 0.0–0.29), but 86.5% of waxy-maize amylopectin, representing the largest macromolecules, was still excluded. The λ_{max} of the iodine–polysaccharide complexes was always constant over the total elution profile of each amylopectin studied. When chromatographed on HW-75, the beta-limit dextrin from waxy-maize amylopectin had a broad elution profile between K_{av} 0.4 and 1.1, with the maximum at K_{av} 0.8 (Fig. 3).

The intrinsic viscosity of acid-hydrolysed amylopectin decreased with time, following a logarithmic relation (Fig. 4). For values of intrinsic viscosities 100 mL/g, λ_{max} and $\overline{c.l.}$ are identical with those of native amylopectins. However, when the period of acid degradation was >5 h, the content of (1→6)-α linkages increased (up

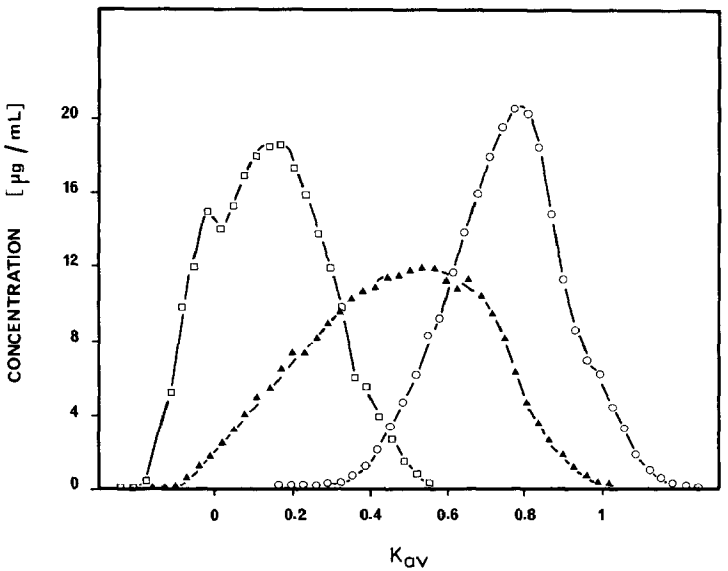

Fig. 3. Elution profiles on HW-75 of native (—□—) and acid-hydrolysed (5 h, —▲—) waxy-maize amylopectin and its beta-limit dextrin (—○—).

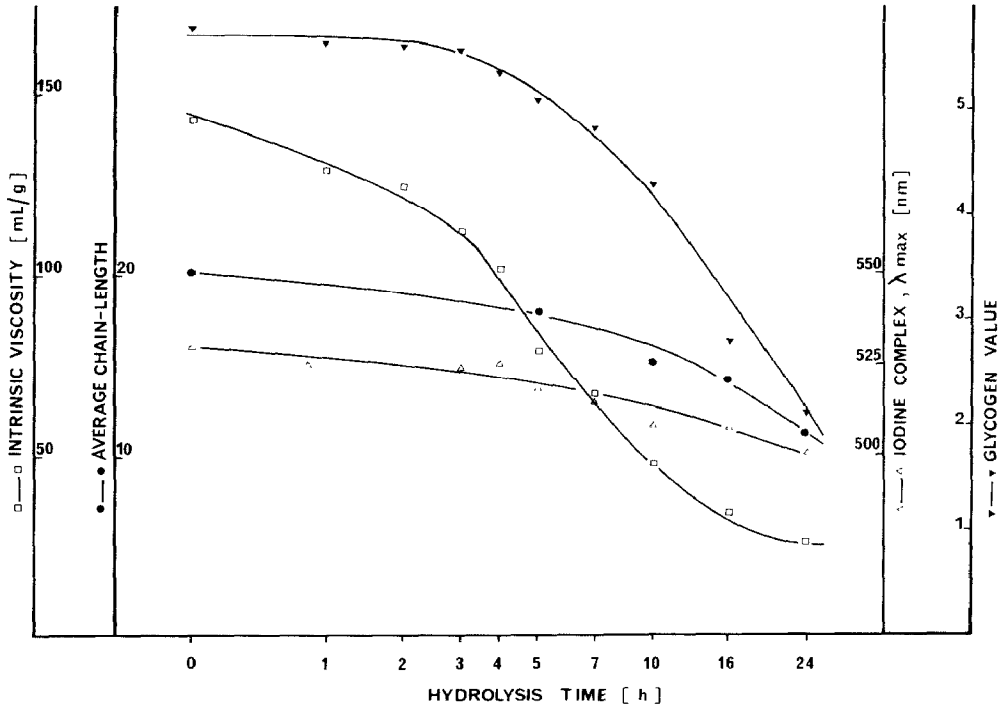

Fig. 4. Characteristics of waxy-maize amylopectin as a function of time of hydrolysis (semi-logarithmic scale): intrinsic viscosity (—□—), λ_{max} (—△—), c.l. (—●—), and g.v. (—▼—)

to 9.1% after hydrolysis for 24 h). Similarly, the peaks in gel chromatography on HW-75 were shifted towards K_{av} 1 (Fig. 3), confirming the decrease in molecular weight as a function of time of hydrolysis; λ_{max} and c.l. decreased to 510 nm and 11, respectively, after hydrolysis of amylopectin for 24 h. Under similar experimental conditions and on the basis of D-glucose oxidase determination, there was no loss of D-glucose in a 0.4% solution, indicating that no reversion reaction had taken place.

Specificity of the reaction of Con A with α-D-glucans. — Two parameters were investigated, namely, time of reaction and concentration of Con A.

At a constant concentration of Con A (0.9 mg/mL), the rate of development of turbidity, when the concentration of glycogen was increased (7–32 μg/mL), reached a maximum within 10 min. Therefore, this time delay was used for subsequent experiments.

At a constant concentration of polysaccharide (smooth-pea amylopectin, 23 μg/mL; oyster glycogen, 36 μg/mL), the turbidity resulting from the interaction with increasing concentration of Con A showed three steps (Fig. 5). Up to 45 μg of Con A/mL for smooth-pea amylopectin and 22.5 μg of Con A/mL for oyster glycogen, no turbidity developed. At higher concentrations of Con A, the turbidity with oyster glycogen increased until a concentration of 0.55 mg of Con A/mL was

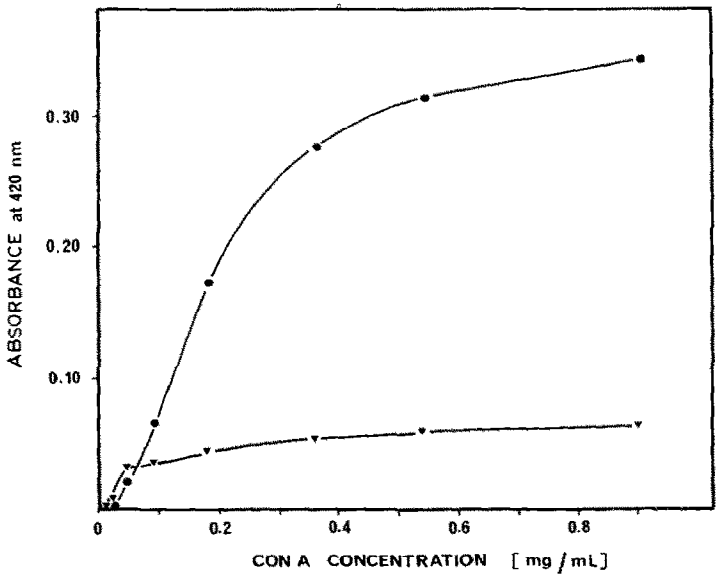

Fig. 5. Turbidity of oyster glycogen (—▼—; final concentration, 10 μg/mL) and smooth-pea amylopectin (—●—; final concentration, 15 μg/mL) as a function of the final concentration of Con A.

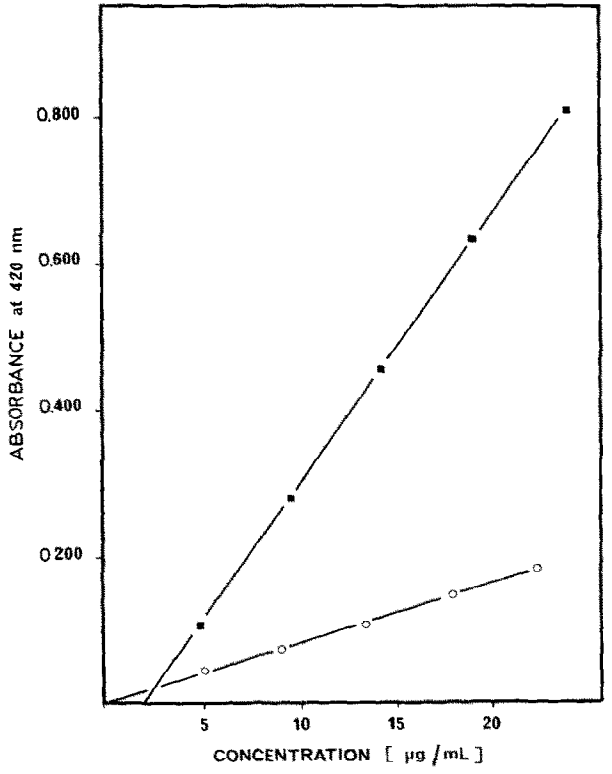

Fig. 6. Relationship between absorbance and concentration of polysaccharide after reaction for 10 min with Con A (0.9 mg/mL): oyster glycogen (—○—), smooth-pea amylopectin (—■—).

reached, beyond which no protein dependence was observed. However, for smooth-pea amylopectin, slight increases occurred up to 0.9 mg of Con A/mL. Therefore, 0.9 mg of Con A/mL appeared to be necessary to obtain maximum interaction of Con A and α-D-glucan, and this concentration was used in subsequent experiments.

The various purified α-D-glucans were tested with Con A, either in their native states, or after modification with enzymes or acid. A linear relationship was observed between absorbance and certain limits of concentration of native polysaccharide (Fig. 6) (*e.g.*, up to 30 μg of glycogen/mL and 20 μg of amylopectin/mL). At higher concentrations of polysaccharide, the assay could not be used due to flocculation of the precipitate. Each native amylopectin had a high g.v. (3.2–6.1), whereas glycogens displayed less-intense reactions (g.v. 1.0–1.3) with Con A (Table I). In contrast, amylose and pullulan did not interact with Con A.

Branched α-D-glucans, when modified by phosphorylase or beta-amylase, displayed reduced g.v. This decrease was only 8.9% for the φ-dextrins and 18–33% for the beta-limit dextrins, compared to the native macromolecules.

The intensity of the interaction of acid-degraded amylopectins from waxy-maize starch was stable for samples whose intrinsic viscosities were >100 mL/g. Below this critical value, the g.v. decreased, following the decline of the intrinsic viscosity (Fig. 4).

The homogeneity of reactive α-D-glucans was studied by analysis of their different fractions, recovered after gel-permeation chromatography. The turbidity reaction requires a concentration of polysaccharide >80 μg/mL. For waxy-maize amylopectin and its beta-limit dextrin, the g.v. decreased from 6.0 to 5.5 along the elution profile on HW-75. Gel-permeation chromatography of oyster glycogen (Fig. 1) on Sepharose CL-2B showed the same change in g.v. from 1.2 (K_{av} 0.51) to 0.8 (K_{av} 0.72).

Influence of Con A on beta-amylolysis. — The initial rate of beta-amylase activity was followed during the first 90 s of hydrolysis using various concentrations of waxy-maize amylopectin (20–900 μg/mL). The values of K_m and V_{max} were deter-

TABLE III

KINETIC CHARACTERISTICS OF BETA-AMYLASE ON WAXY-MAIZE AMYLOPECTIN WITH AND WITHOUT THE PRESENCE OF CON A

pH	*Final Con A concentration (μg/mL)*	K_m *(μg/mL)*	V_{max} *(nM/min)*
7.0	0	77	529
	100	105	569
	300	120	585
	500	152	620
5.2	0	78	1033
	500	81	1152

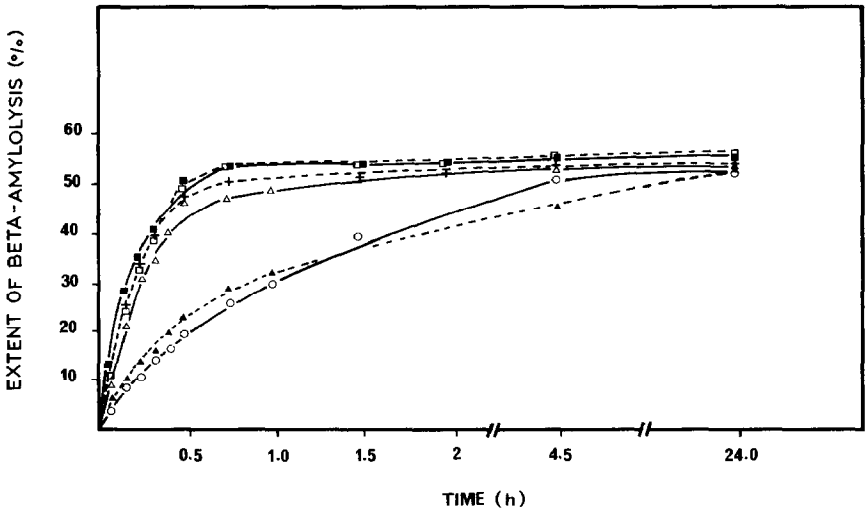

Fig. 7. Beta-amylolysis of waxy-maize amylopectin in the presence of various concentrations (μg/mL) of Con A: pH 5.2, 0 (—■—), 100 (—□—), and 300 (—▲—); pH 7.0, 0 (—+—), 100 (—△—), and 300 (—○—).

mined using a Lineweaver–Burk plot (Table III). At pH 7.0 and without Con A, K_m was 77 μg/mL and V_{max} 529 nM/min/mg of protein. The Hill plot was linear, with a corresponding coefficient in the range 0.9–1.05, which is typical for enzymes following Michaelis kinetics. When the concentration of Con A was increased up to 300 μg/mL, the value of K_m increased to 120 μg/mL, whereas the value of V_{max} was stable. At 500 μg of Con A/mL, the Hill coefficient was 1.4, thereby preventing Michaelis analysis. If maltose (0.025–0.5 mg/mL) was added before the beta-amylase, then K_m was in the range of 70–80 μg/mL.

At pH 5.2 and without Con A, K_m was 78 μg/mL and V_{max} 1033 nM/min. When Con A was added at 500 μg/mL, no change in K_m and V_{max} occurred.

For extended times of hydrolysis at pH 5.2, beta-amylase hydrolysed the bound amylopectin–Con A complexes to the same extent as observed in the absence of Con A. The same observation was made at pH 7.0, although, under these conditions, the rate of beta-amylolysis was lower. At each pH value, the presence of Con A induced a delay in the appearance of maltose during the hydrolysis (Fig. 7).

DISCUSSION

Amylopectins and glycogens have been characterised by their fine structure. Their low capacities of interaction with iodine (λ_{max}, i.b.c.) reflect their branched structure, with short constitutive chains ($\overline{c.l.}$ <25), and prove the macromolecular purity of these fractions[10]. It is thought that each constitutive chain is linked by its reducing end, with its non-reducing chain-end free. An important outcome of this design is the abundance of non-reducing ends per macromolecule, carried at the

end of long flexible chains. Therefore, these molecules are multivalent towards Con A, which binds specifically to terminal non-reducing α-D-glucopyranosyl[1,2] groups. The extents of phosphorolysis and beta-amylolysis agree with previous findings[11,19]. Since the molarity of the resulting ϕ- and beta-limit dextrins corresponds to that of their parent macromolecule, concentration can be expressed on an equal molar basis, without knowledge of the initial molecular weight.

The hydrodynamic volume of these α-D-glucans was investigated by gel-permeation chromatography and measurement of intrinsic viscosity. Sepharose CL-2B, the fractionation range of which is 7×10^4–4×10^7 daltons for globular proteins, may be used either for gel permeation of glycogens and amylose or to detect contaminating amylose in amylopectin fractions. In contrast, HW-75, the fractionation range of which for globular proteins is narrower (5×10^5–5×10^7 daltons), is ideal for the chromatography of amylopectins and allows comparison of relative hydrodynamic volume and polydispersity. Our results show that amylopectins have hydrodynamic volumes larger than those of glycogens. Moreover, no significant correlation could be computed for intrinsic viscosity and chromatographic data for the different amylopectins. This is due to the fact that intrinsic viscosity is only an average value for all molecules, whereas polydispersity is taken into account by gel chromatography.

Recent work[10,27–29] has demonstrated that both glycogen and amylopectin have large molecular weights and are polydisperse. According to Foster[30], the amylopectin molecule in solution is approximately spherical, with the non-reducing chains on the exterior. Although glycogen has a similar conformation, it is more compact, as indicated by its behaviour in gel chromatography and the low value for its intrinsic viscosity. The more open molecular structure of amylopectin agrees with the model proposed by Gunja-Smith *et al.*[31] and Robin *et al.*[9], where half of the internal chains are postulated to have their non-reducing chain-ends in the interior of the macromolecule.

For acid-hydrolysed amylopectin, the degradation reaction can be quantified by measurement of the intrinsic viscosity. As long as the intrinsic viscosity is >100 mL/g (reaction time <5 h), the fine structure is retained, as demonstrated by the constancy of λ_{max} and $\overline{c.l.}$: (1→4)- and (1→6)-α linkages are hydrolysed randomly. However, when acid degradation is prolonged over 5 h, the (1→4)-α linkages are preferentially hydrolysed, leading to an apparent increase in (1→6)-α linkages (*i.e.*, $\overline{c.l.}$ decreases). Having verified that no reversion takes place, the phenomenon is due to different activation energy values for acid hydrolysis of these two covalent bonds[13].

When Con A is present in solution with branched polysaccharides such as amylopectin or glycogen, a network appears progressively (10 min), leading to large particles which scatter light. The experimental conditions described here were very sensitive to the reaction of Con A with α-D-glucans, 5 μg of glycogen/mL or 2 μg of amylopectin/mL being detectable (*cf.* 50–200 μg of polysaccharide/mL in other studies[16,23,33]). The concentration of Con A is a critical parameter. The first

phase, during which no scattering was induced, is explained by the binding of one Con A molecule by one polysaccharide molecule. Under our pH conditions, Con A must be in a tetramer form[34] with a molecular weight of 104,000 at pH 7. Therefore, the four affinity sites of each Con A molecule entrap four chain-ends of the same polysaccharide macromolecule. If the concentration of Con A is increased, multi-molecular complexes appear, giving a sigmoidal curve between absorbance and concentration of Con A. For glycogen, any increase in concentration of Con A above 0.6 mg/mL induces no further increase of turbidity, whereas, for amylopectin, the turbidity increases progressively for the range studied. Assuming Con A to be in a tetramer form, its concentration is of the order 10^{-8} mol/mL or 4×10^{-8} ligand mol/mL. With an average molecular weight of 10^9 for amylopectin and glycogen[10,27,29], the concentration of 10 μg/mL corresponds to 10^{-14} mol/mL. Since $\overline{\text{c.l.}}$ for amylopectin and glycogen is 20 and 12, respectively, non-reducing chain ends are 3×10^{-9} mol/mL and 5×10^{-9} mol/mL, respectively. Therefore, with 10 μg of polysaccharide/mL and 0.9 mg of Con A/mL in the final mixture, there is a slight excess of Con A molecules over branched polysaccharides on a molar basis. If ligand concentration only is taken into account, the concentration of Con A ligands is largely higher than that of glucosyl groups. These observations are consistent with the findings of Hawkins[35], who showed that the greatest rate of turbidity formation occurs at the beginning of the antibody excess zone. With low concentrations of Con A (<20 μg/mL), glycogens are more reactive than amylopectins. At high concentrations (>0.5 mg/mL), this order is reversed, confirming the previous findings of Smith *et al.*[6]. These phenomena are due to the higher hydrodynamic volumes and longer $\overline{\text{e.c.l.}}$ for amylopectin than for glycogen. When the concentration of Con A is too low, the long external chains and the less compact structure of amylopectin make intramolecular reactions easy. As soon as a network appears, the particles with amylopectin are larger, leading to higher g.v. The dependency of the interaction on protein concentration should explain the different results[4,16] obtained in experiments where too little attention has been given to the concentration of Con A.

A major factor controlling the interaction is the $\overline{\text{e.c.l.}}$ of the α-D-glucan. The reduction of the lengths of the external chains is followed by a decrease in g.v. of 10% with phosphorylase and 25% with beta-amylase, confirming previous work on glycogen[32]. When expressed on an equimolar basis, the reduction $\overline{\text{e.c.l.}}$ leads to an increase of g.v. It is well known that beta-amylolysis slightly decreases (25%) the gyration radius of amylopectin[36] as proved also by gel-permeation chromatography on HW-75. However, the higher molar reactivity of limit dextrins must be related to some thermodynamic parameters. A translational activation entropy barrier must be overcome in order that the glucosyl units can reach the Con A sites. Thus, when the non-reducing chain ends of a branched polysaccharide have to find the affinity sites of Con A and stay in the position appropriate for interaction, long chain-length is a negative characteristic, since translational activation entropy increases with increase in chain length.

An important decrease in g.v. was also observed on increasing the time of acid hydrolysis of waxy-maize amylopectin. However, during the first phase of hydrolysis (5 h), the reduction of hydrodynamic volume was not associated with a significant variation of the g.v. Therefore, the hydrodynamic volume should have a limited value, above which any increase does not modify the growing network. When macromolecules are below this critical value, the g.v. decreases greatly as proved also by the relation of g.v. to the elution profile of glycogen. The critical hydrodynamic volume should correspond to special packing of Con A and amylopectin macromolecules during the formation of the network by intermolecular bonds.

Much attention has been given[4,16] to the content of (1→6)-α linkages and its positive correlation with g.v. The degree of branching is not directly involved in the reaction, but is important because any (1→6)-α linkage in amylopectin or glycogen induces a new non-reducing chain end, available for the four Con A sites. Previous results[4] were obtained from macromolecules uncharacterised in terms of either hydrodynamic volume and/or fine structure. In contrast, our work demonstrates that amylopectins, which are less branched than glycogens, are more reactive. Furthermore, their modification by acid or enzymes led to decreases in g.v., although branching is concomitantly increased, confirming the previous results[4,16]. Amylose [homopolymeric (1→4)-α-D-glucan] and pullulan [linear polymer of maltotriose units linked (1→6)-α-, with only one non-reducing chain end per molecule] are unable to create a network by interaction with Con A.

These results demonstrate that hydrodynamic volume and e.c.l. are the major parameters controlling the intensity of the reaction with Con A. Characterisation of the fractions obtained by gel-permeation chromatography of waxy-maize amylopectin, its beta-limit dextrin, and oyster glycogen shows little variation of g.v. as a function of K_{av}, which confirms the constancy of the primary structure, whatever the molecular weight. Con A-amylopectin aggregates are stable complexes, but, as they are not cross-linked by covalent bonds, they can be dissociated easily by decreasing the pH to 2.0. However, even in neutral or slightly acid solutions, these aggregates must be considered as dynamic associations, where non-reducing chain ends can be easily hydrolysed by specific enzymes.

The action of beta-amylase on equilibrated Con A-amylopectin aggregates has been studied. Whereas the value of V_{max} for beta-amylase, acting on pure amylopectin, agrees with previous findings[7,37], the K_m is lower, demonstrating a higher affinity. This is due to the fact that amylopectin presents many more non-reducing chain ends than amylose, the substrate generally used. When Con A is added to the hydrolysis medium, the increase of K_m and the constancy of V_{max} prove the existence of a competitive inhibition. Since Con A cannot interact with beta-amylase, being a pure protein, these results may be interpreted by the combination of Con A with the non-reducing chain ends of the polysaccharide, which compete with the binding of beta-amylase. As soon as beta-amylolysis takes place, maltose is released and binds to Con A in such a way as to mask the affinity site.

This explanation agrees with the fact that D-glucose and maltose inhibit the Con A–α-D-glucan interaction[7,38]. In this situation, amylopectin becomes more accessible, allowing hydrolysis by beta-amylase. Thus, extended hydrolysis by beta-amylase has the same limit with and without Con A, as already observed by Doyle *et al.*[32]. If Con A is present in excess, the Con A–polysaccharide complex leads to a non-Michaelis kinetics, by formation of a heterogeneous phase.

Our work is essentially a phenomenological characterisation of the formation of bonds between two large molecules, namely Con A and branched polysaccharides. A quantitative description in terms of thermodynamic parameters (free energies and equilibrium constants)[39] will be carried out from the present knowledge of the structural requirements for Con A–α-D-glucan interactions.

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