Protein-Carbohydrate Interaction

A TURBIDIMETRIC STUDY OF THE INTERACTION OF CONCAVALIN A WITH AMYLOPECTIN AND GLYCOGEN AND SOME OF THEIR ENZYMIC AND CHEMICAL DEGRADATION PRODUCTS

By E. E. SMITH,* ZEENAT H. GUNJA SMITH* AND I. J. GOLDSTEIN†

*Department of Biochemistry, State University of New York at Buffalo,
Buffalo, N.Y. 14214, U.S.A.*

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The interaction of the plant protein concanavalin A with glycogen and amylopectin was studied. The protein concentration-dependency of the interaction and the effect on the interaction of various enzymic and chemical degradations of the polysaccharides were investigated by using a turbidimetric technique. The results indicate that, in addition to certain structural requirements, the molecular weight of the polysaccharides and the concentration of protein are important factors controlling the extent of interaction. The observed similarity of the interaction of polysaccharides with concanavalin A to the more specific polysaccharide-protein interactions indicates that it should provide a useful model system for the study of these interactions in general.

A large number of plant proteins are capable of agglutinating certain types of erythrocytes and bacterial cells. A few of these plant proteins, also termed phytohaemagglutinins and lectins, also have been shown to interact, with the formation of an insoluble precipitate, with the related blood-group or bacterial mucopolysaccharides. Many of the interactions reported are highly specific in nature and have proved to be of great interest in the fields of immunochemistry and immunology (Boyd, 1963).

Sumner & Howell (1936) reported that concanavalin A, a globulin present in the seeds of *Canavalia ensiformis* (jack bean), agglutinated the erythrocytes of a number of mammalian species. The agglutination of a suspension of starch granules was also demonstrated, as was the capacity of the protein to interact with glycogen in solution to form an insoluble precipitate. More recently Cifonelli, Montgomery & Smith (1956) observed that the extent of interaction with glycogen, as measured by a turbidimetric technique, is dependent on the origin of the glycogen involved. This observation led to the introduction by these workers of the concept of 'glycogen value', in which glycogens are assigned numerical values on the basis of their interaction with concanavalin A, under defined conditions, relative to that of an

equal weight of rabbit liver glycogen, which is assigned a value of 1.0. A gross relationship between 'glycogen value' and the degree of branching of a large number of glycogen specimens was reported by Manners & Wright (1962). Goldstein, Hollerman & Merrick (1965a) reported a similar interaction of concanavalin A with dextrans and it was observed that the apparent degree of branching of the dextrans was also probably related to their 'glycogen value'. However, the extent of interaction of the dextrans was markedly dependent on the concentration of concanavalin A, and it was demonstrated that by increasing the amount of protein present amylopectin also interacted to form an insoluble precipitate. Under the conditions of the earlier workers (Cifonelli *et al.* 1956; Manners & Wright, 1962) the interaction of amylopectin, a molecule closely related to that of glycogen, was not observed.

The similarity of the interaction of glycogen with concanavalin A to that which occurs between antigen and antibody (Goldstein *et al.* 1965a) makes this interaction of great interest. The ease of isolation of large amounts of relatively pure protein, and the availability of enzymes with which the structure of glycogen and amylopectin can be modified, makes it an almost ideal model system for investigations into the mechanism of complex-formation between polysaccharide and protein.

The protein concentration-dependency of the interaction of glycogen and amylopectin with concanavalin A, as measured by a turbidimetric method, has now been studied. The interaction of

* Present address: Department of Biochemistry, University of Miami School of Medicine, Miami, Fla. 33152, U.S.A.

† Present address: Department of Biological Chemistry, University of Michigan, Ann Arbor, Mich. 48104, U.S.A.

several glycogen samples and of the products obtained by controlled enzymic and chemical degradation of glycogen was investigated. In addition, the relationship of the molecular size of these polysaccharides to the extent of their interaction with concanavalin A is discussed.

MATERIALS AND METHODS

A three-times-crystallized preparation of *Bacillus subtilis* α -amylase was a gift from Dr P. A. Allen; a sample of glucoamylase dried on Celite powder (1580 units/g.; A. E. Staley Manufacturing Co., Decatur, Ill., U.S.A.) was kindly presented by Dr A. W. Turner. Sweet-potato β -amylase (three-times crystallized) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A., Pronase (grade B) was from Calbiochem, Los Angeles, Calif., U.S.A., and O.K. Corn Starch was a sample from A. E. Staley Manufacturing Co. Yeast mannan (*Saccharomyces cerevisiae*) and Floridean starch were a gift from Professor S. Peat, and dextran NRRL B-1355-S was obtained through the courtesy of Dr A. Jeanes. Shellfish and oyster glycogen were purchased from Eastman Kodak, Rochester, N.Y., U.S.A.

Reducing sugars were determined by the method of Schaffer & Hartmann (1921) and polysaccharide concentrations by the phenol-H₂SO₄ method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

Isolation of glycogen

Rabbit liver glycogen. The liver of a freshly killed rabbit was rapidly homogenized in ice-water and the homogenate was divided into three portions. A 30% (w/v) KOH solution was added to the first portion to bring the final concentration to 20% (w/v). The solution was heated at 100° for 1 hr. and the glycogen was isolated and purified by repeated precipitation with ethanol (2 vol.). An equal volume of ice-cold 10% (w/v) trichloroacetic acid was added to the second portion and the mixture was ground with sand for 5 min. and centrifuged. The sediment was twice re-extracted with ice-cold 5% (w/v) trichloroacetic acid and the glycogen was isolated and purified by an ethanol precipitation as described above. All procedures in this extraction were carried out at 4°. The cold-water extraction method of Bueding & Orrell (1964) was used to isolate the glycogen from the third portion of the original homogenate.

Rat liver glycogen. The livers of three freshly killed rats were homogenized in ice-water and glycogen was isolated from portions of the homogenate by the three extraction procedures noted above.

Degradation of polysaccharides

β -Amylolysis. Alkali-extracted rabbit liver glycogen, commercial oyster glycogen and amylopectin (O.K. Corn Starch) were degraded with β -amylase by the method of Whelan (1964). The total polysaccharide concentration of each digest was determined by complete acidic hydrolysis of a portion, followed by assay of reducing sugar (Pirt & Whelan, 1951). Samples of the three digests were removed at intervals, heated at 100° for 3 min. and the extent of

β -amylolysis of each sample was calculated from the increase in reducing power. When no further increase in reducing power of the three digests was observed they were heat-inactivated and dialysed against distilled water for 24 hr. Denatured protein was removed by filtration and filtrates were evaporated to a small volume under reduced pressure and freeze-dried.

Glucoamylolysis. Alkali-extracted rabbit liver glycogen, commercial oyster glycogen and commercial shellfish glycogen (500 mg.) were each dissolved in 0.02 M-sodium acetate-HCl buffer, pH 4.8 (200 ml.). A suspension of glucoamylase powder (2 ml.; 10 mg. of Celite powder/ml.; 32 units) was added to each solution and samples (20 ml.) were immediately removed and heat-inactivated. The digests were kept at room temperature and samples (20 ml.) were removed at intervals and heat-inactivated at 100° for 5 min. The extent of degradation was determined as described for the β -amylase degradation and the degraded glycogens were similarly dialysed and isolated as freeze-dried powders.

α -Amylolysis. A solution of the β -amylase limit dextrin of alkali-extracted rabbit liver glycogen (50 mg./5 ml. of water) was mixed with a solution of crystalline *Bacillus subtilis* α -amylase (0.01 ml., 2 mg./ml.). A portion (0.7 ml.) was immediately heat-inactivated. The digest was kept at room temperature and several samples (0.7 ml.) were removed over a period of 60 min. and similarly inactivated. The samples were filtered and dialysed against distilled water for 24 hr. and the polysaccharide concentration of each dialysed solution was determined by the phenol-H₂SO₄ method. These solutions were used directly in the concanavalin A interaction.

Treatment with alkali. Commercial oyster glycogen (50 mg.) was dissolved in 5.6 N-KOH solution (50 ml.). The solution was placed in a boiling-water bath and a vigorous stream of CO₂-free air was bubbled through. Samples (5 ml.) were removed at intervals over 7.5 hr. and neutralized with 3 N-H₂SO₄. After filtration each sample was dialysed against distilled water for 24 hr. and freeze-dried.

Acidic hydrolysis. Commercial oyster glycogen (1.0 g.) was dissolved in 5% (w/v) trichloroacetic acid (100 ml.) at room temperature. Samples (20 ml.) removed at intervals over 186 hr. were neutralized with 3 N-NaOH solution. The neutral solutions were dialysed against distilled water for 24 hr. and freeze-dried.

Centrifugal fractionation of alkali-degraded glycogen. A solution (1 mg./ml.) of alkali-degraded oyster glycogen (5 hr. sample) was centrifuged (Spinco model L, rotor 40) at 115000 g for 6.5 hr. at 4°. By using a Pasteur pipette with an upturned tip, a sample (1 ml.) was carefully removed from near the surface of the centrifuged solution. The remaining solution was carefully siphoned off until only 1 ml. of solution remained at the bottom. The concentrations of glycogen in the top and bottom fractions were determined by the phenol-H₂SO₄ method and each sample was tested for its interaction with concanavalin A.

Concanavalin A extract

Concanavalin A was extracted from jack-bean meal by the method of Sumner & Howell (1936) and the purified preparation was dissolved in saturated NaCl solution and stored at 3°.

Precipitation assays

The required amount of concanavalin A (total protein determined as by Lowry, Rosebrough, Farr & Randall, 1951) in 2 ml. of half-saturated NaCl solution containing 2 μ moles of sodium acetate-HCl buffer, pH 5.2, was added to a series of matched test tubes (10 cm. $\times$ 1.3 cm.) containing an aqueous solution (1.0 ml.) of polysaccharide. The contents of each tube (total volume 3.0 ml.) were stirred with a rod and kept at 25° for exactly 10 min. The extinction was then read at 420 m μ in a Bausch and Lomb Spectronic 20 spectrophotometer. The extinctions of two series of tubes, in which the polysaccharide solution was replaced with water or the protein solution with half-saturated NaCl solution, were subtracted from the test readings to correct for the turbidity of these solutions.

Two-dimensional agar-gel diffusion studies of glycogen samples were carried out under the conditions described by Goldstein & So (1965).

RESULTS

The turbidity resulting from the interaction of different concentrations of amylopectin and of alkali-extracted rabbit liver glycogen in the presence of two concentrations of concanavalin A (4 mg. and 1 mg. of total protein/3 ml. respectively) are shown in Fig. 1. At the higher protein concentration interaction was evident for both polysaccharides, but that of glycogen was so high as to be outside the range of the spectrophotometer and has therefore not been included in Fig. 1. When the concentration of protein was decreased to 1 mg./3 ml. the interaction of glycogen appreciably diminished, whereas that of amylopectin decreased to so low a level that it could not be recorded.

The protein concentration-dependence of the turbidimetric assay was further confirmed when the interaction of a constant concentration (0.25 mg./3 ml.) of five different polysaccharides was measured in the presence of various concentrations of concanavalin A extract (Fig. 2): with the exception of Floridean starch, the relative order of interaction of the polysaccharides at low protein concentrations is completely reversed at the higher protein concentration (cf. 0.5 and 4.0 mg. of total protein/3 ml.).

Cifonelli *et al.* (1956) reported that, on an equal-weight basis, the increase in interaction with concanavalin A of glycogen β -limit dextrin over that of the parent glycogen is approximately proportional to the degree of β -amylolysis, and it was therefore concluded that the interaction of the dextrin molecule is similar to that of the undegraded glycogen molecule. Direct comparisons of the interactions of equimolar concentrations of alkali-extracted rabbit liver glycogen (0.25 mg./3 ml.) and its limit dextrin (0.137 mg./3 ml. calculated from degree of β -amylolysis) and of amylopectin (0.25 mg./3 ml.) and its β -limit dextrin

Fig. 1. Extinction (at 420 m μ) of the precipitates formed by the interaction of various amounts of amylopectin in the presence of concanavalin A (4 mg. of total protein) (●) and of various amounts of alkali-extracted rabbit liver glycogen in the presence of concanavalin A (1 mg. of total protein) (○). The total volume was 3 ml.

Fig. 2. Extinction (at 420 m μ) of the precipitates formed by the interaction of 0.25 mg. of various polysaccharides in the presence of various amounts of concanavalin A: □, yeast mannan; ●, amylopectin; ■, dextran NRRL B-1355-S; ○, alkali-extracted rabbit liver glycogen, ×, Floridean starch. The total volume was 3 ml.

(0.115 mg./3 ml.) were therefore made in the presence of various concentrations of concanavalin A extract. It is evident from the extinction curves obtained (Fig. 3) that in both cases the interaction of the parent polysaccharide was more extensive than that of the dextrin, particularly at high protein concentrations. The greater maximum extinction

Fig. 3. Extinction (at 420 m μ) of the precipitates formed by the interaction of amylopectin (0.25 mg.) and an equimolar amount (0.115 mg.) of its β -amylase limit dextrin and of alkali-extracted rabbit liver glycogen (0.25 mg.) and an equimolar amount (0.137 mg.) of its β -amylase limit dextrin in the presence of various amounts of concanavalin A extract: ●, amylopectin; ■, amylopectin β -limit dextrin; ○, glycogen; □, glycogen β -limit dextrin. The total volume was 3 ml.

Table 1. *Extinctions (at 420 m μ) of the maximum precipitation formed by the interaction of (a) equal weights (0.20 mg.) and (b) equimolar quantities of β -amylase-degraded rabbit liver and oyster glycogens in the presence of excess of concanavalin A extract*

Values are taken from curves as in Fig. 4.

β -Amylolysis (%)	Rat liver glycogen		Oyster glycogen	
	(a)	(b)	(a)	(b)
0	0.26	0.26*	0.24	0.24*
12	—	—	0.26	0.23
13	0.28	0.25	—	—
21	0.28	0.22	0.26	0.20
25	—	—	0.27	0.20
36	0.30	0.20	—	—
41	0.30	0.19	0.31	0.19
45	0.32	0.18	—	—

* These values are taken from column (a).

observed for amylopectin in this experiment is due to the use of a different concanavalin A extract of greater specific activity than that of the extract used in the experiments illustrated in Fig. 2. Once this maximum extinction is reached the interaction of the polysaccharide ceases to be dependent on the protein concentration, i.e. no further increase in

Fig. 4. Extinction (at 420 m μ) of the precipitates formed by the interaction of 0.20 mg. of the products of glucoamylase degradation of alkali-extracted rabbit liver glycogen in the presence of various amounts of concanavalin A extract: ●, 0% degradation; ■, 21% degradation; ▲, 42% degradation; ▼, 51% degradation; ×, 71% degradation. The total volume was 3 ml.

Table 2. *Maximum extinctions (at 420 m μ) of the precipitates formed by the interaction of equal weights (0.20 mg.) of glucoamylase-degraded oyster and shellfish glycogens in the presence of excess of concanavalin A extract*

Values are taken from curves as in Fig. 4.

Glucoamylase degradation (%)	Oyster glycogen	Shellfish glycogen
0	0.25	0.24
11	—	0.24
21	0.27	—
24	—	0.26
34	—	0.29
39	0.30	—
41	—	0.31
51	0.35	—
55	—	0.35
62	—	0.40
71	0.46	—
74	—	0.58

extinction occurs in the presence of additional protein. This portion of the curve was therefore used to examine the interaction with concanavalin A of a number of dextrans obtained by the use of different methods of controlled degradation of several glycogen samples.

The maximum interactions of the degradation products obtained by β -amylase treatment of two glycogen samples are shown in Table 1. With increasing degradation the maximum interaction of

Fig. 5. Extinction (at 420 mμ) of the maximum precipitation formed in the presence of excess of concanavalin A extract by 0.2 mg. of the non-diffusible products of various times of degradation of: (a) oyster glycogen with 5% (w/v) trichloroacetic acid at room temperature (Δ); (b) oyster glycogen with 5.6N-KOH solution at 100° (□); (c) rabbit liver glycogen β-limit dextrin with α-amylase (○). The total volume was 3 ml.

equal weights of the dextrans rose significantly, whereas that of equimolar amounts fell. The interactions of equal weights of glucoamylase-degraded rabbit liver glycogen are shown in Fig. 4, and the maximum extinctions of equal weights of glucoamylase-degraded oyster and shellfish glycogens determined from similar curves are shown in Table 2. In each case the maximum extinction showed an increase with degradation. An increase in the maximum interaction of equal weights of the glycogen degradation products is also apparent with increasing time of alkali or acid treatment. The further degradation of a β-amylase limit dextrin by a small amount of *Bacillus subtilis* α-amylase is again accompanied by increasing maximum extinctions when equal weights of these dextrans are allowed to interact with various amounts of concanavalin A extract (Fig. 5).

High- and low-molecular-weight polysaccharide fractions were isolated by ultracentrifugation from the non-diffusible polydisperse degradation products resulting from a 5 hr. hot-alkali treatment of oyster glycogen. Each fraction was allowed to interact with concanavalin A over a range of protein concentrations (Fig. 6). The lighter fraction,

Fig. 6. Extinction (at 420 mμ) of the precipitates formed in the presence of various amounts of concanavalin A by 0.1 mg. of two centrifugal fractions of alkali-degraded oyster glycogen: ○, sedimented polysaccharide; ●, non-sedimented polysaccharide. The total volume was 3 ml.

Table 3. Extinctions (at 420 mμ) of the maximum precipitation formed by the interaction of equal weights (0.20 mg.) of rat and rabbit liver glycogen samples extracted by three different methods, and of two standard-molecular-weight glycogens, in the presence of excess of concanavalin A extract

The preparation of the glycogen samples is described in the text. Values are taken from curves as in Fig. 4.

Source	Extraction method	E ₄₂₀
Rat	Water	0.18
Rat	Trichloroacetic acid	0.21
Rat	KOH	0.27
Rabbit	Water	0.18
Rabbit	Trichloroacetic acid	0.19
Rabbit	KOH	0.28
Phytoglycogen (mol. wt. 20 × 10 ⁶)	—	0.18
Pectoral-muscle glycogen (mol. wt. 2.5 × 10 ⁶)	—	0.18

though exhibiting a relatively poor interaction at the lower concanavalin A concentrations, showed a rapid increase in extinction with increasing protein concentration, eventually reaching a significantly higher extinction than that of the heavier polysaccharide fraction (cf. Fig. 2).

Equal weights of rat and rabbit liver glycogen samples, isolated by three different extraction procedures, were allowed to interact with various concentrations of concanavalin A extract and the maximum extinctions obtained were compared

Fig. 7. (a) Glucoamylase-degraded glycogen-concanavalin A precipitation in agar gel. Portions (0.1 ml.) of degraded alkali-extracted rabbit liver glycogen solution (1.0 mg./ml.) were added to the peripheral wells: A, undegraded; B, 21% degraded; C, 42% degraded; D, 71% degraded; E, undegraded (0.05 ml.) + 71% degraded (0.05 ml.); F, 21% degraded (0.05 ml.) + 71% degraded (0.05 ml.); concanavalin A extract (0.1 ml., 3 mg./ml.) was added to the centre well and the precipitation bands were allowed to develop for 24 hr. at room temperature. (b) Rat liver glycogen-concanavalin A precipitation in agar gel. Portions (0.1 ml.) of the polysaccharide solutions (1 mg./ml.) were added to the peripheral wells: A, alkali-extracted glycogen; B, trichloroacetic acid-extracted glycogen; C, cold-water-extracted glycogen; D, alkali-extracted glycogen (0.05 ml.) + cold-water-extracted glycogen (0.05 ml.); E, phytoglycogen (mol. wt. 20×10^6); F, pectoral-muscle glycogen (mol. wt. 2.5×10^6); concanavalin A extract (0.1 ml., 3 mg./ml.) was added to the centre well and the precipitation bands were allowed to develop for 24 hr. at room temperature. (c) Alkali-degraded oyster glycogen-concanavalin A precipitation in agar gel. Portions (0.1 ml.) of the polysaccharide solutions were added to the peripheral wells: A, undegraded oyster glycogen; B, alkali-treated (5 hr.) oyster glycogen; C, alkali-treated (7.5 hr.) oyster glycogen; D, water-extracted rat liver glycogen; E, water-extracted rat liver glycogen (0.05 ml.) + alkali-treated (5 hr.) oyster glycogen (0.05 ml.); F, water-extracted rat liver glycogen + alkali-treated (7.5 hr.) oyster glycogen. Concanavalin A extract (0.1 ml., 6 mg./ml.) was added to centre well and precipitation bands were allowed to develop overnight at room temperature.

with those of two glycogen samples of known molecular weights (Table 3). The increase in extinction values of the extracted glycogen samples reflects the order of the extent of degradation of the products that results when water, trichloroacetic acid and hot-alkali extraction procedures respectively are used (Orrell & Bueding, 1964). Unexpectedly, the pectoral-muscle glycogen gave the same extinction value as the high-molecular-weight phytoglycogen. The explanation for this is unknown and the low extinction value for the muscle glycogen could not be confirmed owing to shortage of material.

The concanavalin A precipitation patterns of the extracted and standard glycogen samples and also of a number of the degraded glycogens were examined by two-dimensional diffusion in agar gel. It is apparent from Fig. 7(a) that increasing glucoamylase degradation of oyster glycogen results in the formation of precipitation bands at an increasing distance from the polysaccharide wells (wells A, B, C and D). Mixtures of two of these fractions formed only a single precipitation band (wells E and F).

The increasing molecular weight of the alkali-, trichloroacetic acid- and water-extracted rat liver glycogens (Table 3) is reflected by the decreasing distance of the outer edge of the precipitation bands

from the polysaccharide wells (A, B and C; Fig. 7b). This difference, however, is too small to allow the resolution of a mixture of alkali- and water-extracted glycogens into two precipitation bands (well D). The relative positions of the precipitation bands formed by the high-molecular-weight phytoglycogen (mol. wt. 20×10^6) and the pectoral-muscle glycogen (mol. wt. 2.5×10^6) is consistent with their different molecular sizes (wells E and F). The diffuse nature of the precipitation band formed between the pectoral-muscle glycogen and concanavalin A (well F) suggests this glycogen to be quite polydisperse. A similar pattern involving increasing degradation with alkali is shown in Fig. 7(c) (wells A, B and C). Though resolution of a mixture of water-extracted glycogen and alkali-degraded (5 hr.) glycogen was not observed (well E), two bands were formed corresponding to the components of a mixture of water-extracted glycogen and a glycogen sample subjected to the alkaline degradation for 7.5 hr. (well F). The glycogen samples extracted from rabbit liver by the three different procedures were tested in the same way and provided results similar to those obtained with the rat liver glycogens (Fig. 7b).

The rabbit liver glycogen samples were tested for their interaction in the presence of a constant

Fig. 8. Extinction (at $420m\mu$) of the precipitates formed in the presence of concanavalin A extract (0.3mg. of total protein) by various amounts of glycogen and degraded glycogen preparations: (a) □, cold-water-extracted rabbit liver glycogen; ●, trichloroacetic acid-extracted rabbit liver glycogen; ○, alkali-extracted rabbit liver glycogen; ■, β -amylase limit dextrin of alkali-extracted rabbit liver glycogen; (b) ●, oyster glycogen; Δ, alkali-degraded (2 hr.) oyster glycogen; □, β -amylase limit dextrin of oyster glycogen. The total volume was 3 ml.

concentration of concanavalin A extract (0.3mg./3ml.) but with various concentrations of polysaccharide. Figs. 8(a) and 8(b) show that, under these conditions, the least-degraded glycogen samples show the greatest maximum turbidities. Thus degradation of the glycogen molecule, whether resulting from the mode of extraction, treatment with hot alkali or from enzymic action, resulted in a decrease of the maximum constant turbidity. This is the opposite effect to that observed in the presence of relatively high concentrations of concanavalin A extracts.

DISCUSSION

The relationship between glycogen structure and its interaction with concanavalin A has been investigated in a number of Laboratories. Cifonelli *et al.* (1956) postulated that the intact inner branches of the glycogen molecule are involved in the formation of the protein-polysaccharide complex, and Manners & Wright (1962) reported an approximate relationship between the degree of branching and the relative interaction with concanavalin A ('glycogen value') of a large number of glycogen specimens. The failure of amylopectin to form a precipitate with concanavalin A under standard assay conditions, compared with the strong interaction of several samples of horse muscle glycogens possessing similar degrees of branching, led Manners & Wright (1962) to conclude that there are fundamental structural differences between glycogen and amylopectin in addition to their degree of branching.

It should be noted that the latter workers observed the initial formation of a precipitate when 10mg. of potato amylopectin was added to a concanavalin A extract, the precipitate dissolving at the end of 30 min. We interpret this as indicating that the localized concentrations of amylopectin and concanavalin A were compatible with precipitate formation. However, the total relative concentrations of these two components were such that there was an excess of amylopectin, i.e. the extreme end of the zone of amylopectin excess of the precipitation curve was attained and hence the precipitate dissolved.

On the basis of inhibition studies conducted in this Laboratory (Goldstein, Hollerman & Smith, 1965b; Smith & Goldstein, 1967) and studies with synthetic carbohydrate-protein conjugates (Goldstein & Iyer, 1966) it was concluded that the concanavalin A molecule interacts with glycogen and amylopectin at the terminal non-reducing ends of external branches rather than at the internal branches. Though no precipitate was reported for amylopectin and a number of dextrans under the assay conditions used by the earlier workers (however, see above), interaction with higher concentrations of concanavalin A resulted in the formation of an insoluble precipitate (Goldstein *et al.* 1965a). The similarity of the interactions of amylopectin and glycogen is shown in Fig. 1, both curves possessing the characteristics of a specific interaction.

The protein concentration-dependency of the interaction of a number of polysaccharides is well illustrated in Fig. 2, which shows that the turbidimetric response with increasing protein concentration gave different curves for each polysaccharide. Thus it is observed that completely different 'glycogen values' would be obtained for these

polysaccharides if the assays were conducted at two different protein concentrations. The use in the standard 'glycogen-value' assay of crude concanavalin A extracts of unknown concentration must therefore be avoided, as extracts made in different Laboratories will probably vary widely in their content of active protein. [Use of the pure protein would obviate this difficulty (Agrawal & Goldstein, 1965).] An example of this variation in activity is apparent in Fig. 3, where a new extract was used. That this extract is much more active than that used in Fig. 2 is evident from the rapid rise of the amylopectin curve to give a maximum value in the presence of 4 mg. of total protein/3 ml., whereas the amylopectin curve in Fig. 2 does not reach a maximum value, even in the presence of 12 mg. of protein/3 ml. Once this maximum extinction value is reached the interaction is not influenced by further increase in protein concentration.

The similarity of the initial portions of the two curves obtained for equimolar amounts of amylopectin and its β -amylase limit dextrin (Fig. 3) may indicate that the number of non-reducing terminal chain ends is related to the turbidimetric response at relatively low concanavalin A concentrations. The number of chain ends is the same in the dextrin and the parent polysaccharide molecule. Insofar as there is a greater number of dextrin molecules per unit weight, the higher extinction per unit weight of the β -amylase limit dextrin over that of the parent amylopectin is consistent with the interaction of concanavalin A at the chain ends of the polysaccharide molecules (Goldstein *et al.* 1965a). The greater extinction of the amylopectin curve over that of an equal weight of the more highly branched glycogen, however, indicates that in the presence of a high concentration of protein a factor (or factors) other than the degree of branching is operative.

The significantly lower maximum extinction values (Fig. 3) of the two limit dextrin curves, compared with those of their parent polysaccharides, suggest that molecular size may be involved. In fact, it would be expected that the higher-molecular-weight parent polysaccharides would make a greater contribution to the mass of the precipitate than their corresponding β -limit dextrans. This is indicated further by the fall in the maximum extinction reached by equimolar amounts of the glycogen dextrans obtained by increasing degrees of β -amylolysis of rabbit liver and oyster glycogens (Table 1). The concomitant rise in the values obtained by interaction of equal weights of these dextrans reflects the increasing number of molecules present with a corresponding decrease in molecular weight.

It could be argued that the progressive increase in the maximum extinction values reached in the interaction of equal weights of these dextrans

reflects the increase in degree of branching that accompanies β -amylase degradation of glycogen. That this is unlikely is shown by the similar increase in the maximum extinction observed when the interactions of equal weights of the non-diffusible products obtained by several methods of degradation of the various glycogen samples were tested.

Glucosylase, unlike β -amylase, is also capable of hydrolysing the α -(1 $\rightarrow$ 6)-bonds that form the branch points in the glycogen molecule, resulting in a more complete degradation of this polysaccharide. Hydrolysis of more than 60% of the glucosidic linkages must involve the cleavage of some α -(1 $\rightarrow$ 6)-bonds, yet the dextrans obtained on 60–70% hydrolysis still exhibit an increase in maximum extinction with degradation (Table 2 and Fig. 4).

Increasing time of degradation with the essentially random hydrolysis catalysed by α -amylase, hot alkali or acid also resulted in increasing maximum extinction values (Fig. 5). The decreasing size of the molecule accompanying such degradations would therefore seem to be implicated, and this conclusion is supported by the greater interaction at high concanavalin A concentrations of the lighter non-sedimented fraction of degraded glycogen compared with that of the sedimented fraction (Fig. 6).

Cabantchik & Schramm (1965), however, reported the isolation of macromolecules that contained 22–25% of (1 $\rightarrow$ 6)-branch linkages after mild α -amylolysis of glycogen. Undegraded glycogen contains only 6–10% of (1 $\rightarrow$ 6)-linkages (Manners & Wright, 1962). It is therefore possible that the high-molecular-weight non-diffusible products of the various degradations used in this investigation contain an increased number of (1 $\rightarrow$ 6)-linkages. Glycogen may consist of a wide range of molecules of different degrees of branching, or there may be an uneven distribution of branches within the molecule itself so that it contains regions much more densely branched than is indicated by the degree of branching of the molecule as a whole. In both cases a preferential hydrolysis of the less densely branched structures would lead to the formation of residual high-molecular-weight molecules corresponding to the macromolecules described by Cabantchik & Schramm (1965). β -Amylase and glucosylase act at the non-reducing chain ends of glycogen in a stepwise manner with the release of maltose and glucose respectively. β -Amylase is specific for α -(1 $\rightarrow$ 4)-linkages and can neither hydrolyse nor by-pass α -(1 $\rightarrow$ 6)-linkages, and its action is therefore terminated as it approaches the outermost branch linkages of the glycogen molecule. The hydrolysis of (1 $\rightarrow$ 4)-linkages only thus results in an increasing proportion of (1 $\rightarrow$ 6)-linkages in the residual dextrans as β -amylolysis progresses. Though glucosylase is able to hydrolyse α -(1 $\rightarrow$ 6)-bonds the rate of

hydrolysis of these bonds is much lower than that of the α -(1 $\rightarrow$ 4)-linkages. Therefore the preferential hydrolysis of the less densely branched structures of glycogen by glucoamylase would also lead to an enrichment of (1 $\rightarrow$ 6)-branch linkages in the residual high-molecular-weight molecules. Similarly, in the essentially random hydrolysis of glycogen catalysed by α -amylase and acid, the specificity of α -amylase for the α -(1 $\rightarrow$ 4)-linkage and the relative resistance of the (1 $\rightarrow$ 6)-linkage to hydrolysis under acidic conditions may also result in an enrichment of (1 $\rightarrow$ 6)-linkages in the high-molecular-weight products obtained by the two methods of degradation.

The possibility therefore remains that the increase in the extinction maximum of the degraded glycogen-protein interaction observed for increasing degradation is related to a parallel increase in the degree of branching of the products. However, the similarity of the extinction curves of amylopectin (Fig. 2) and degraded glycogen (Fig. 6) relative to that of undegraded glycogen argues against this conclusion. An increase in branching with degradation of glycogen is unlikely to result in the extinction curve of the degradation products becoming more similar to the extinction curve of amylopectin, a molecule that has a lower degree of branching than glycogen. Further, the decreasing molecular size of glycogen samples extracted with cold water, trichloroacetic acid and boiling potassium hydroxide solution (Orrell & Bueding, 1964) is confirmed by their relative rates of diffusion on agar plates (Figs. 7b and 7c). No significant difference in the degree of branching of these molecules would be expected and therefore the increasing maximum extinctions in the presence of excess of concanavalin A extract (Fig. 3) and the decreasing extinctions at a relatively low protein concentration (Fig. 8a) exhibited by equal weights of these samples must be related to differences in their molecular weight. The formation of two precipitation bands by a mixture of water-extracted rat liver glycogen and an alkali-treated (7.5 hr.) oyster glycogen in the agar diffusion (Fig. 7c, well F) may be interpreted to support further the importance of molecular size in the concanavalin A interaction. Thus the rapidly diffusing degraded glycogen will move against the concentration gradient of concanavalin A diffusing in the opposite direction. The time necessary for the degraded glycogen to reach a region of sufficiently high protein concentration for precipitation to occur is sufficient to allow a low concentration of concanavalin A to diffuse into the slow-moving high-molecular-weight glycogen and cause precipitation to take place. Thus the two precipitation bands are the result of the different protein concentration requirements of the two glycogen samples rather than a difference in their specificities in the interaction.

The importance of molecular size in the interaction of polysaccharides with concanavalin A indicated in these studies therefore makes it unlikely that 'glycogen values' (Cifonelli *et al.* 1956) obtained from a standard turbidimetric assay, in which neither the protein concentration nor the mode of extraction of the glycogen assayed are subject to control, can be validly interpreted in terms of molecular structure alone.

The similarity of the effects of branching frequency (cf. glycogen and amylopectin; Fig. 2) and molecular size (Fig. 6) on the shape of the concanavalin A extinction curves suggests the possibility that these effects may be related. It can be calculated that a glycogen molecule of mol.wt. 2×10^6 and an average chain length of 12 glucose units possesses about 1000 non-reducing chain ends, all of which are potentially capable of interacting with the receptor sites of concanavalin A (Goldstein *et al.* 1965a). Steric considerations, however, indicate that the chain ends are in high excess and that the number of protein molecules that can interact at any one time is a function, not of the number of chain ends, but rather of the total molecular surface area of the polysaccharide molecule. Thus in the presence of excess of protein the entire surface of the glycogen molecule may be covered by concanavalin A molecules. This is analogous to the theory of antigen-antibody

(a) Relative total surface area or (b) relative number of molecules

Fig. 9. Extinction (at 420 m μ) of the maximum precipitation formed in the presence of excess of concanavalin A by 0.2 mg. of glucoamylase-degraded rabbit liver glycogen plotted against: (a) calculated relative total surface area $[1/(1-x)]^\dagger$, where x is the degree of degradation of glycogen of the molecules present (●); (b) relative number of molecules $[1/(1-x)]$, where x is the degree of degradation of glycogen present (○).

complex-formation in the region of antibody excess put forward by Pauling (1940). The increase in the relative total surface area of a given weight of polysaccharide, which would result from a decrease either in degree of branching or in molecular weight, may explain the relatively high maximum interactions with excess of protein of amylopectin and the degraded glycogens.

Support for this theory can be found in the report that relatively low-molecular-weight clinical dextrans precipitate more antibody in the region of antibody excess than does an equal weight of the higher-molecular-weight native dextran (Kabat & Berg, 1953). However, though a reasonably good linear relationship is obtained between maximum extinction and the relative total surface area of equal weights of the glucoamylase-degraded glycogens (calculated on the basis of an ideal spherical molecule), an even closer relationship was found between the extinction and the relative number of polysaccharide molecules present (Fig. 9). This is obviously inconsistent with the fall in the maximum extinctions obtained with equimolar amounts of the progressively degraded β -amylase glycogen dextrans.

The smaller amounts of a horse serum antibody precipitated at a relatively low antibody/polysaccharide ratio by the lower-molecular-weight fractions of a synthetic polyglucan (Heidelberger & Aisenberg, 1953) is analogous to the inferior interaction of the degraded glycogens in the presence of low concentrations of concanavalin A, and provides further evidence for the similarity of the concanavalin A interaction to the antibody-antigen system. At low concanavalin A concentrations, however, the number of variables involved in the interaction makes interpretation of the relative extinctions in terms of the molecular dimensions and structure of glycogen even more complicated, and therefore beyond the scope of the turbidimetric technique.

Recent studies in this Laboratory (Poretz & Goldstein, 1968) indicate the time-dependency of the turbidimetric response of polysaccharide-concanavalin A interaction, such dependence being a function of the region of the equivalence curve that is under investigation. For this reason most of our subsequent studies have been conducted with the quantitative precipitin procedure (So & Goldstein, 1967a). However, it has been demonstrated that inhibition studies, whether conducted by the turbidimetric method (Goldstein *et al.* 1965b; Smith & Goldstein, 1967) or by the quantitative precipita-

tion technique involving nitrogen analysis of washed specific precipitates (So & Goldstein, 1967b; Poretz & Goldstein, 1968), give identical results.

The above results strongly suggest that the application of quantitative protein and polysaccharide analytical methods to the concanavalin A-glycogen interaction may provide data that would be invaluable in the elucidation of specific polysaccharide-protein interactions in general.

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