ether–methanol mixture and dried *in vacuo* at 100° to give authentic *cis*-2-dimethylaminocyclopentanol methiodide, m.p. 146–147°.

trans-2-Dimethylaminocyclopentanol Methiodide (VII).—A solution of 14 g. of purified cyclopentene oxide in 40 ml. of ethanol was mixed with a threefold excess of 33% aqueous dimethylamine, sealed into a glass bomb and heated at 120–130° (oil-bath) for 24 hours. After cooling, the bomb's contents were transferred to a separatory funnel with the aid of about 100 ml. of water, saturated with salt and extracted with ether. The ether extract was dried over anhydrous magnesium sulfate and fractionated to yield 12.7 g. of *trans*-2-dimethylaminocyclopentanol, b.p. 106° (16 mm.). Three grams of this aminoalcohol was then added to a solution of 13 g. of methyl iodide in 100 ml. of absolute ether and the resulting mixture shaken overnight. Precipitation of the methiodide was quite rapid, and a threefold recrystallization from ether–methanol mixture gave the pure product, m.p. 207–208°.

Anal. Calcd. for $C_8H_{18}NOI$: C, 35.44; H, 6.69; N, 5.17. Found: C, 35.14; H, 6.76; N, 4.96.

trans-2-Dimethylaminocyclopentyl Acetate Methiodide (VIII).—A solution of 5 g. of *trans*-2-dimethylaminocyclopentanol in 75 ml. of anhydrous ether was continuously stirred and bubbled with ketene for a period of four hours. The ethereal solution was then filtered, mixed with 20 g. of methyl iodide and shaken mechanically overnight. The precipitated methiodide was recrystallized repeatedly from methanol–ether mixture; m.p. 166–167°.

Anal. Calcd. for $C_{10}H_{20}NO_2I$: C, 38.35; H, 6.44; N, 4.47. Found: C, 38.48; H, 6.31; N, 4.61.

Enzymatic Rate Determinations.—The equipment and techniques employed were essentially those of a previous study.[3] A standard phosphate buffer of *p*H 7.3 and containing 0.01 *M* Mg(II) ion was used in both inhibitor and substrate work, with acetylcholine chloride serving as substrate in evaluation of inhibitor strength. The titration cell was thermostated at 25.12 ± 0.03°. The stock enzyme solution[13] from which aliquots were drawn and diluted for kinetic experiments assayed at 2.7×10^5 μmole acetylcholine hydrolyzed/hr./mg. protein, with initial substrate concentrations in the range $1\text{–}10 \times 10^{-3}$ *M* being employed.

Substrates and inhibitors were freshly recrystallized samples, with solutions being prepared and refrigerated just before use. Rate runs were restricted to points within the first 5–7% of reaction to avoid complications due to build-up of the weakly inhibitory alcohol products and corrected for non-enzymatically catalyzed hydrolysis of substrate. Rates were in general reproducible to ±5%. In the plots of v/v_I *vs.* inhibitor concentration to obtain K_I values, the maximum uncertainties in K_I noted in Table I were estimated from each respective least-squares fit of the data.

Acknowledgments.—We are indebted to Dr. H. S. Polin for his generous aid in procurement of electric eel tissue and its initial extracts and to Mr. J. Hockstad for valuable technical assistance.

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The Reaction between Concanavalin-A and Glycogen[1]

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Glycogens from various biological sources give a precipitin reaction with concanavalin-A, a globulin from Jack Bean meal. It is found that certain glycogens can be differentiated from others by their capacity to react with concanavalin-A and that native starches including waxy maize as well as the A- and B-fractions of various starches differ from glycogens by their failure to give the reaction. Since removal of the outer branches of glycogen by β-amylase results in an increase in the precipitin reaction, there is reason to believe that the reaction involves primarily the inner portions of the molecules. The fact that all amylaceous polysaccharides do not precipitate concanavalin-A equally well thus suggests that they may not have identical inner structures.

Basic similarities among glycogens from different sources have been established by the use of various chemical and enzymatic procedures. Thus, methylation[2–5] of glycogens has indicated average unit chains of twelve α-D-glucose units linked through positions 1 and 4 and joined to each other through position 1 of the "reducing residue" to position 6 of a glucose residue in another chain. There is also evidence concerning the presence of other linkages.[6–9]

Periodate oxidation techniques[7,10,12–14] confirmed the presence of one terminal unit for approximately 12 D-glucose residues, though it has been claimed that certain glycogen samples may contain one terminal unit per chain length of six residues[14,15] while in other instances the number of end groups per chain has been reported as being as low as one in 18[10,11,14,16] or even one in 23 residues.[16]

An enzymatic method[17–19] has been employed to determine the ratio of branch points to end-groups and the position of the branch point in each unit chain. The results from this method were said to indicate that a regular series of glycogens exists with chain lengths varying from 10 or less units to approximately 18 D-glucose units.

Glycogen has been shown to give a precipitate when treated with concanavalin-A,[20] a globulin extracted from Jack Bean meal. The work herein

(1) Paper No. 3297, Scientific Journal Series, Minnesota Agricultural Experiment Station. This work constitutes part of a thesis submitted by J. A. Cifonelli to the Graduate Faculty of the University of Minnesota in partial fulfillment for the degree of Ph.D., 1953.

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shows that this protein–carbohydrate reaction which can be measured turbidimetrically[21] may be used in certain cases to distinguish glycogen from different sources.

Experimental

Preparation of Concanavalin-A Solution and Determination of Glycogen Values.—Concanavalin-A solution is prepared by the extraction of Jack Bean meal[22] with 2% saline according to the method previously described.[21] The solution, stable for several months under refrigeration, may be filtered if necessary prior to use.

For the turbidimetric determination of glycogen values (G.V.), 9 ml. of the concanavalin-A solution is added to 1 ml. of solution containing 0.1 to 1.0 mg. of glycogen. The "glycogen value" (G.V.) is determined by interpolating on a standard curve, prepared by the use of purified human liver or rabbit liver glycogen which shows a G.V. of 1.00 as a standard, the weight of standard glycogen which corresponds to the optical density shown by 1 mg. of the glycogen being tested.[21]

Isolation and Purification of Glycogens.—In general, glycogen was extracted from tissues by treatment with boiling 30% (w./w.) potassium hydroxide for 2 hr., followed by acidification with glacial acetic acid and precipitation with two volumes of ethanol. The crude precipitate so obtained was dissolved in 5% aqueous trichloroacetic acid. After keeping at 5° overnight, the mixture was centrifuged and the supernatant liquid treated with two volumes of ethanol. The precipitate was dissolved in water and reprecipitated with two volumes of ethanol. The glycogen was washed successively with ethanol, ether, light petroleum ether and dried *in vacuo*.

Every effort was made to ensure that the samples of glycogen were freed from proteinaceous and lipid impurities. In certain instances (glycogens from salmon liver, dogfish liver, Ascaris and sweet corn) purification was effected by acetylation (see Table II).

The glycogen was freshly precipitated from aqueous solution with ethanol, washed with ethanol (once), dissolved in pyridine and treated with one volume of acetic anhydride. The acetylation proceeded with the evolution of heat but no external cooling was applied. After keeping overnight, the mixture was poured with stirring into water. The acetate was filtered and washed with water and ethanol. Deacetylation proceeded easily when an acetone solution of the acetate was heated for 10 minutes with an equal volume of 20% potassium hydroxide with stirring. At the completion of the reaction the lower alkali layer, containing the regenerated glycogen, assumed an opalescent appearance. The solution was cooled, acidified slightly with acetic acid and treated with two volumes of ethanol. The precipitate was dissolved in water and reprecipitated with ethanol. The precipitation was repeated and the glycogen washed successively with ethanol, ether, light petroleum ether and dried *in vacuo* at 56°.

Glycogen from rabbit liver, human liver, dogfish liver, salmon liver, rabbit leg muscle and from yeast was purified by fractional precipitation from aqueous solution with increasing amounts of ethanol. Each fraction was redissolved in water and the solution poured with stirring into ethanol (4 vol.). The precipitated glycogen was washed with ethanol, with ether and dried *in vacuo* (see Table II).

Amylolysis of Glycogens.—α-Amylolysis was effected by the use of diluted saliva (1:25) buffered at *p*H 7 with 0.05 *M* phosphate.

β-Dextrins of various glycogen samples were obtained by using β-amylase from soy bean flour buffered at *p*H 4.6 with acetate to give a final acetate concentration of 0.01 *M*. The soy bean amylase was stirred with 200 parts by weight of buffer and after standing overnight, the mixture was filtered and 1 ml. of this solution was used per 70–80 mg. of the glycogen. For the preparation of the limit dextrins the amylolysis was allowed to proceed for 24 hr. or more and the dextrins were isolated by precipitation with 2 volumes of alcohol.

Reducing sugars produced during amylolysis were determined by the 3,5-dinitrosalicylic acid method.[23]

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TABLE I

THE GLYCOGEN VALUES (G.V.) OF GLYCOGENS FROM DIFFERENT SOURCES

Source of glycogen	G.V.
Muscle	
Rabbit leg	1.00
Rabbit abdominal	1.00
Fish	1.20
Rat	1.45
Liver	
Ox (14 hours after death)	0.95
Rabbit	1.00
Fish, bullhead	1.00
Fish, bass	1.00
Horse, normal	1.05
Guinea pig	1.05
Rabbit (alloxan treated)	1.10
Chicken	1.00
Turkey	1.10
Horse, fasted and fed D-galactose	1.15
Rabbit, fasted and fed D-galactose	1.15
Fish, Northern pike	1.15
Fish, salmon[a]	1.15
Frog	1.20
Rabbit, 18 unit type	1.25
Fish, bullhead, fasted	1.30
Dogfish[a]	1.30
Human 1, von Gierke disease[b]	1.30
Human 2, von Gierke disease	1.35
Human 3, von Gierke disease	1.27
Rat	1.40
Bat, hibernating	1.70
Miscellaneous	
Oyster	1.15
Hair, rabbit	1.45
Wild bee larvae	1.00
Helminth, *Moniezi expansa*	1.25
Helminth, *Ascaris lumbricoides*[a]	1.35
Protozoan, *Tetrahymena pyriformis*	1.35
Baker's yeast[a]	2.85
Fungus, *Pleurotius ostreatius*	1.00
Sweet corn (phytoglycogen)[a]	1.05
Other polyglucosans[c]	
Starch, corn	0.00
Starch, waxy corn	.00
Amylose, corn	.00
Amylopectin, corn[d]	.00
Starch, potato	.00
Amylose, potato	.00
Amylopectin, potato	.00
Laminarin	.00
Dextran (*Leuconostoc mesenteroides*, B-512)	.00

[a] Purified through the acetate. [b] The yield was 10% of the wet weight of the liver. [c] Synthetic amylose, made from D-glucose 1-phosphate with potato phosphorylase and synthetic amylopectin made with a mixture of potato phosphorylase (P-enzyme) and potato Q-enzyme, gave no reaction. The amylose-like polysaccharide synthesized from glucose 1-phosphate by the action of muscle phosphorylase in the absence of a glycogen "starter" likewise gave no reaction with concanavalin-A. [d] The branched chain, B-fractions, derived from wheat, sago, tapioca, rice and Easter lily starch failed to give a precipitin reaction.

Results and Discussion

From the results summarized in Table I, it is evident that glycogens from various biological

sources precipitate with concanavalin-A, although certain samples differ from others in precipitating capacity (G.V.). The glycogens from muscle and liver of different species show similar ranges of values.

Of the muscle glycogens, that from the rabbit showed the lowest value and that from the rat the highest. Among the liver glycogens, those from starved animals frequently showed a higher G.V. than material from the corresponding normal animal. Of interest is the observation that, from hibernating bat livers, a glycogen was obtained in very small yield which showed a higher G.V. than did any of the other liver glycogens examined. A comparison of "normal" human liver glycogen with that obtained from three patients with von Gierke's disease reveals significantly higher values for the von Gierke glycogens.[17,24-26]

The glycogens obtained from the invertebrates likewise differed in their capacity to precipitate with concanavalin-A. Glycogens from the helminths (*Ascaris lumbricoides*[12] and *Moniezi expansa*)[2] and from the protozoan (*Tetrahymena pyriformis*)[27] all show values greater than that for normal rabbit liver glycogen.

Of all the glycogens examined, yeast glycogen showed by far the greatest G.V. It is to be noted that yeast gum also is precipitated by concanavalin-A solution, but the large G.V. shown by the yeast glycogen is not due to the presence of yeast gum as an impurity, since salivary α-amylase completely destroys the capacity of the yeast glycogen to be precipitated by the concanavalin-A solution; yeast gum is not affected by α-amylase treatment.[21]

The concanavalin-A reaction supports the suggestion that sweet corn (*Zea mays*) glycogen shows the properties of animal glycogen,[28,29] a normal G.V. being found. Furthermore, β-amylolysis proceeds to a limit which is normal for animal glycogens[30] and the β-limit dextrin so obtained shows an increased G.V. consistent with values shown by various animal glycogen β-limit dextrins (see Table III).

The polysaccharide obtained by alkali extraction of rabbit hair[31,32] also appears to be a true glycogen as judged both by the concanavalin-A reaction and by β-amylolysis; its G.V. approaches the upper limit noted for animal glycogens, only the hibernating bat liver glycogen showing a greater G.V. A sample of glycogen isolated from the fungus *Pleurotus ostreatus* was also found to give a G.V. of 1.0 although its rotation was low.

It has been suggested that there is no sharp distinction between the glycogen and the amylopectin groups of polysaccharides[17-19] and that the main difference between the two groups of polysaccharides concerns the proportion of branch points, the ratio of inner to outer branch length remaining approximately the same.[14,15,33,34] Waxy corn starch and potato starch as well as the A- and B-fractions of other starches showed no reaction with concanavalin-A. It was also found that dextran produced by the organism *Leuconostoc mesenteroides* NRRL-512B and laminarin gave no precipitate with concanavalin-A.

It is evident from a study of the fractions obtained by acetylation or fractional precipitation with ethanol of certain glycogen samples that the G.V. increases with increasing optical rotation (Table II). It would appear from these results and those of α-amylolysis that the concanavalin precipitation reaction depends on the glycogen in a preparation and is not due to an impurity.

Of interest also is the fact that the liver glycogen from a patient with von Gierke's disease appeared to be of uniform purity (Table II). All fractions gave clear solutions with normal specific rotations

TABLE II

THE "GLYCOGEN VALUES" OF CERTAIN GLYCOGEN SAMPLES AFTER TREATMENT BY ETHANOLIC FRACTIONATION AND BY ACETYLATION

Source of glycogen	Fraction no.	$[\alpha]_D^{(H_2O)}$	Glycogen value
I. Purification by ethanolic fractionation			
Baker's yeast	I	+180°	1.80
	II	+182°	2.10
	III	+ 11°	0.35
	IV	+ 39°	0.25
	Original	+162°	..
Rabbit leg muscle	I	+ 45°	0.60
	II	+ 90°	0.50
	III	+185°	1.00
	IV	+ 90°	0.50
	Original	+155°	0.80
Rabbit liver	I	+185°	0.95
	II	+195°	1.00
	Original	+180°	0.90
Human liver	I	(Too turbid to read)	0.50
	II	+190°	1.00
	III	- 20°	0.05
	Original	+175°	0.90
Human liver, von Gierke disease	I	+195°	1.30
	II	+195°	1.30
	III	+195°	1.40
Dogfish liver	I	....	1.05
	II	....	1.30
	III	....	1.50
II. Purification via acetylation			
Sweet corn, original		+185°	0.90
Sweet corn, after being purified *via* acetate		+201°	1.05
Salmon liver, original		+170°	0.75
Salmon liver, after being purified *via* acetate		+191°	1.15

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and a higher than normal precipitin reaction (G.V.) with concanavalin-A.

Treatment of various liver and muscle glycogens with salivary α-amylase resulted in the rapid disappearance of any precipitating ability with concanavalin-A solution.

The β-limit dextrin of waxy corn starch produced no turbidity when treated with concanavalin-A solution. This observation is significant in the light of the various reports that glycogen and amylopectin are very similar, the main distinction being in their chain lengths.[14,17-19,33,34] Variation of the exterior chain length is of little or no consequence in the concanavalin-A:glycogen reaction for the data in Table III show that removal of the outer branches by β-amylolysis leaves a β-dextrin showing an increase in G.V. approximately proportional to the degree of hydrolysis.

TABLE III

THE "GLYCOGEN VALUES" (G.V.) OF β-LIMIT DEXTRINS PREPARED FROM VARIOUS SAMPLES OF GLYCOGEN

Source of glycogen	G.V. Unhydrolyzed	G.V. After amylolysis	Hydrolysis,[a] %
Human liver	1.00	1.30	35
Rabbit liver	1.00	1.35	41
Rabbit hair	1.45	2.25	48
Northern pike liver	1.15	1.45	33.5
Sweet corn (*Zea mays*)	1.05	1.45	49
Baker's yeast[b]	3.0	4.5	45
Waxy corn starch	0.00	0.00	..

[a] Reducing sugars (as maltose) determined with 3,5-dinitrosalicylic acid[23] after maximum hydrolysis with β-amylase. (Incubation time, 24 hr.). [b] Purified by elution from carbon-celite column.[35]

Additional information bearing on the structural requirements for the glycogen concanavalin-A reaction is provided by the fact that methylated glycogen (from mussels, *Mytilus edulis*) had no precipitating ability, while periodate oxidation of rabbit liver and Northern pike liver glycogens, a reaction known to attack the outer before the inner branches of the molecule,[36] caused a progressive decrease in their precipitating ability.

It would appear, therefore, that the concanavalin-A complex involves not only the intact inner branches of the glycogen molecule but also the hydroxyl groups of the molecule. Further investigations into the structural requirements of the polysaccharide concanavalin-A reaction and its use in the study of the fine structure of polysaccharides is in progress.[37]

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The Reaction of Concanavalin-A with Mucopolysaccharides[1]

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It is known that concanavalin-A, a globulin protein of jack bean meal, forms an insoluble complex with yeast mannan[2,3] and glycogen[4,5] in aqueous solution. By the use of this reaction it is possible to analyze quantitatively for these two materials[3] and also to differentiate the glycogen polyglucosans from the amylopectins,[5] a division which has been variously suggested to be chemically unsound.[6,7]

A study of the reaction has now been extended to the hexosaminehexuronic acid polymers, which include heparin, mucoitinsulfuric acid, chondroitinsulfuric acid and hyaluronic acid. The presence of such carbohydrate polymers in cartilage, connective tissue and blood group substances among products of animal origin suggested that an application of the concanavalin reaction to the qualitative and quantitative study of these compounds would be desirable.[8] Heparin has been found to give a precipitation reaction with concanavalin-A. Upon quantitative analysis[5] it was found that sodium heparinate[9] had 50% more precipitating ability than normal human liver glycogen, which throughout these investigations[5] has been taken as a

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(9) The authors wish to express their thanks to Dr. M. L. Wolfrom, for providing samples of sodium heparinate[10] and beef lung galactogen.

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