HETEROGENIETY OF GLYCOGEN

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R. G. Hansen
Major professor

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ABSTRACT

HETEROGENEITY OF GLYCOGEN

by John Hoffman Nordin

Several examples of variations in chemical composition and structure in glycogen have been reported in the literature. An unusual specimen of glycogen has been obtained from the livers of chicks fed toxic levels of galactose. This glycogen has been shown to contain small but significant amounts of galactose. It has been subjected to exhaustive purification procedures which include precipitation with the antibody concanavalin A, acetylation and deacetylation. The galactose is not removed by these procedures. Comparison of this glycogen with samples from various other sources indicate that the galactose is not a result of an impurity or an artifact arising during hydrolysis of the glycogen. Fractional precipitation of this polysaccharide followed by chromatography of the hydrolyzed fractions indicate galactose is associated with each fraction obtained. Fractionation of glycogen with added free radioactive galactose results in the complete removal of this sugar during the early stages of purification.

The identity of the galactose has been confirmed by co-chromatography with radioactive galactose and by preparation of galactose methyl phenyl hydrazone from acid hydrolyzates of the glycogen.

HETEROGENEITY OF GLYCOGEN

By

John Hoffman Nordin

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ERRATUM: Please read Heterogeneity for Heterogeniety throughout text.

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To My Family

VITA

John H. Nordin was born on October 11, 1934, at Chicago, Illinois where he was graduated from Taft High School. During the period from 1952 until 1956, he attended the University of Illinois where he received a B.S. degree in Agriculture. His graduate studies were pursued in the Department of Dairy Science of that same institution until 1958 when he transferred to Michigan State University to complete the requirements for the degree of Doctor of Philosophy in the Biochemistry Department. A postdoctoral position at the Department of Agricultural Biochemistry, University of Minnesota was recently accepted.

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INTRODUCTION

When a 15% galactose diet is fed to newly hatched chicks, toxicity symptoms soon develop which include convulsive seizures that often result in death. Previous work in this field indicates that the carbohydrate metabolism of affected birds is severely altered. Examination of the acid soluble nucleotides of their livers indicates that uridine diphosphate hexose metabolism is disturbed. Normally UDPG[1] constitutes about 70% of the total UDP hexose present, while UDPGal constitutes the remaining 30%. Livers from galactose toxic chicks, on the other hand, contain double the amount of UDP hexose and further 95 to 100% of this is UDPGal. The effect of this apparent lack of UDPG is conceivably of serious consequence since this compound is an important glucosyl donor in many biochemical reactions.

Since UDPG functions as a glucosyl donor in glycogen biosynthesis, limitations in the pool size of this compound might be expected to be reflected in glycogen synthesis. Some preliminary indications pointed to alterations of glycogen composition of rat tissues where galactose was fed. However, it appears in birds that liver glycogen levels are not significantly altered as a result of feeding galactose. Since the

[1]The following abbreviations are used: UDPG, uridine diphosphate glucose; UDPGal, uridine diphosphate galactose; UDP hexose, mixture of UDPG and UDPGal; G-1-P, glucose-1-phosphate; Gal-1-P, galactose-1-phosphate; Pi, inorganic phosphate.

phosphorylase pathway of glycogen metabolism does not require UDPG and is reversible, it may be the principal pathway of glycogen biosynthesis in the chicken.

Large quantities of free galactose and its phosphorylated derivatives acculumate in blood and other tissues of chicks fed galactose. The possibility exists that there may be a swamping effect on some biochemical processes. With this in mind, glycogen from chicks fed toxic levels of galactose was examined and found to contain galactose in significant amounts. This occurrence of galactose in what heretofore has been regarded as a homopolymer of glucose may result from the following means: 1. UDPGal may serve as a glycosyl donor. 2. Gal-1-P might act as a glycosyl donor in the phosphorylase pathway. 3. Galactose might be incorporated in a purely chemical reaction without the mediation of any enzyme.

This alteration of glycogen structure might have profound effects on other metabolic transformations. Evidence is accumulating which suggests that other modifications of glycogen exist. These modifications are reflected as unusual repeating units, linkage groups or polymer structure and the presence of sugars other than glucose in the glycogen. The more significant findings are as follows:

1. The presence of $(1 \rightarrow 3)$ linkages is indicated in some glycogens. A small amount of 3-O-α-D-glucopyranosyl-D-glucopyranose (nigerose) has been isolated from beef glycogen as a crystalline derivative. Periodate oxidation studies on several other glycogens support this finding.

2. Isomaltotriose, O-α-D-glucopyranosyl (1 ⟶ 6)-O-α-D-glucopyranosyl (1 ⟶ 6)-D-glucopyranose has also been obtained from beef glycogen. This unusual repeating unit is not characteristic of the classical glycogen molecule (Figure 1).

3. Maltulose, 4-O-α-D-glucopyranosyl-D-fructose, has been isolated from pregnant rabbit liver glycogen hydrolyzates.

4. Glycogens with unusual branching and outer chain character are reported when animals are fed sucrose or galactose. This condition is also found in the various human glycogen storage diseases.

5. Electrophoresis of many different glycogens shows them each to be composed of two distinct molecular species as evidenced by mobility. This indicates that a high degree of heterogeniety exists in a supposedly pure homopolymer.

The biochemical significance of most of the above work is unknown. The cause(s) and effect(s) of each of these abberations in molecular structure may reflect important aspects of polysaccharide specificity. It may be mentioned that similar structural modifications in starches have also been reported.

It is the purpose of this communication to report the occurrence of small amounts of galactose in highly purified liver glycogen of galactose toxic chicks. This galactose has been isolated as a crystalline derivative and the derivative characterized.

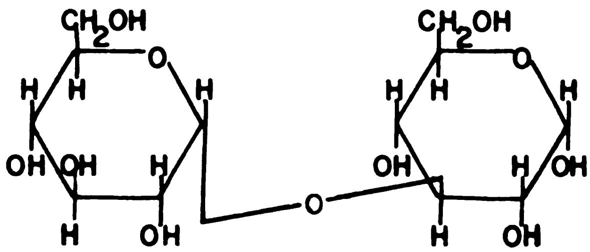
Figure 1.

Structures of three oligosaccharides isolated from partial glycogen hydrolyzates.

A. Nigerose, 3-O-α-D-glucopyranosyl-D-glucopyranose.

B. Isomaltotriose, O-α-D-glucopyranosyl (1 ⟶ 6) O-α-D-glucopyranosyl (1⟶ 6) D-glucopyranose.

C. Maltulose, 4-O-α-D-glucopyranosyl-D-fructose (The applicable configuration of fructose has not been confirmed but is shown in the furanose form).



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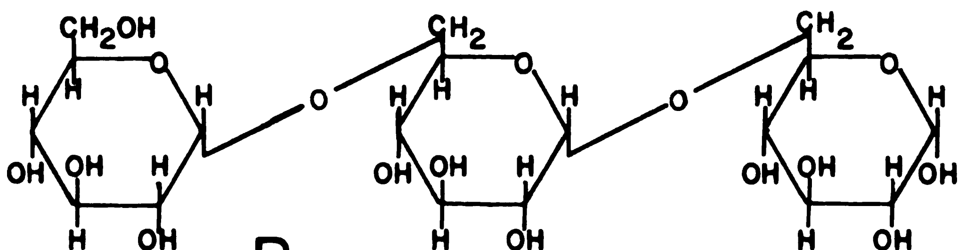

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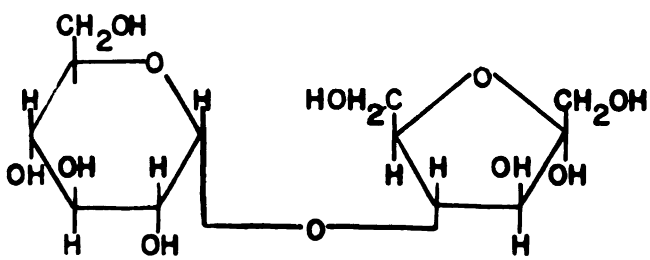

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LITERATURE REVIEW

General Structure of Glycogen

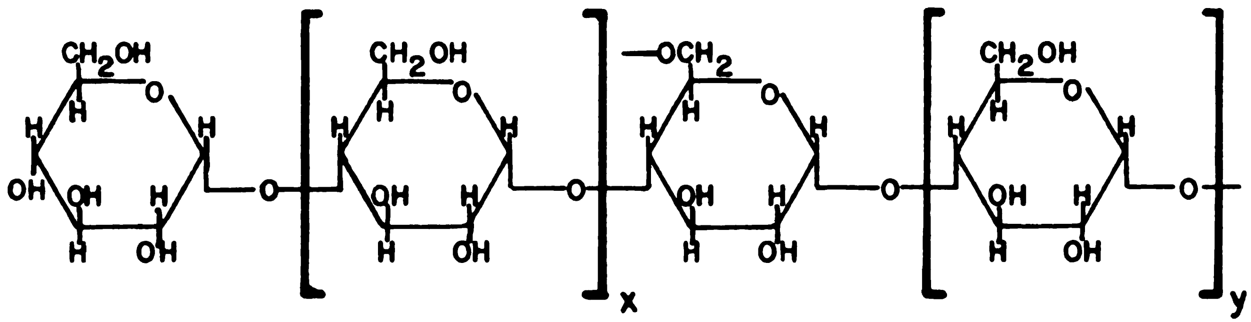
Glycogen, the principle carbohydrate reserve in liver, muscle, and other tissues was first isolated by Bernard from dog liver (1). Structural studies by Haworth and others (2-5) showed glycogen to be a branched high molecular weight carbohydrate polymer composed entirely of D-glucopyranose units. These studies indicated that in the average repeating unit of the molecule about eleven glucopyranose units are joined by $1 \rightarrow 4$-α-D linkages while one molecule is joined through C6 to form a branch point (Figure 2). Periodate oxidation experiments (6-8) on glycogen and enzymatic analyses (9,10) support Haworth's work.

The molecular weight of most glycogens appears to be approximately 10^7. There is some discrepancy in the magnitude of this general value. This may result from the method of isolation of the polymer and method of molecular weight determination. When sedimentation data are employed, the range in molecular weights is about 2×10^6 to 8×10^6 (11,12) whether alkaline digestion (KOH glycogens) or cold trichloroacetic acid (TCA glycogens) extraction are employed in the isolation of the glycogen. However, when light scattering measurements are used, TCA glycogens appear in some cases to be very heavy while glycogens isolated by alkali digestion have much lower molecular weights. One group reports (13) molecular weights in the range of 2×10^6 to 5×10^6 for KOH glycogens while TCA glycogens gave values of from 40×10^6 to 159×10^6. Other workers however, fail to

Figure 2

Classical Chemical Structure of an Average Repeating Unit of Glycogen

AVERAGE REPEATING UNIT OF GLYCOGEN



$(x + y = 10)$

obtain this difference with light scattering. Bryce et al. (14) determined molecular weights on 23 different glycogen samples. They could find no extreme variation in molecular weights. KOH glycogens and glycogens prepared by extraction with water showed molecular weights in the range of 3×10^6 to 9×10^6.

The wide variation that occurs has been attributed both to impurity of the glycogen when isolated by TCA (15) and to degradation of the glycogen during alkali extraction (13). Stetten and Katzen in a recent study (16) indicated that degradation of TCA glycogens occurs when it is subjected to hot alkali. The release of small amounts of isosaccharinic acid from the glycogen has been shown to accompany the degradation.

Metabolism of Glycogen

Four known enzymes catalize the reactions responsible for the major interconversions of glycogen. Phosphorylase (17) catalizes the reversible reaction

$$\text{G-1-P} + (\text{Glucosyl})_n \rightleftharpoons (\text{Glucosyl})_{n+1} + \text{Pi}.$$

The reaction product is formed by the addition of an α-(1 ⟶ 4) linked glucopyranose unit to a non-reducing end group of glycogen. Branched chain polysaccharides such as starch and glycogen act as primers for phosphorylase while the linear polymer amylose is without effect. There appears to be a definite primer requirement for rapid conversion of added glucose-1-phosphate to glycogen. Branching of the glycogen molecule is brought about by the enzyme amylo 1,4 ⟶ 1,6 transglucosidase (10). It causes the transfer of a group of α-(1 ⟶ 4) linked units to C6 of another glucose unit forming a branch point. The continued action of these two enzymes leads to the formation of the highly branched polysaccharide.

In the catabolism of the glycogen molecule, phosphorylase cleaves single α-(1 $\longrightarrow$ 4) glucose units up to a branch point yielding a limit dextrin while forming G-1-P. In order to cleave the α-(1 $\longrightarrow$ 6) linkages at the branch points the enzyme amylo 1 $\longrightarrow$ 6 glucosidase is required (9). Hydrolysis of this bond permits continued phosphorolysis of the limit dextrin to the next branch point.

Illingworth, Brown and Cori have recently reported (18,19) the occurrence of slow <u>denovo</u> synthesis of glycogen from G-1-P without a glycogen primer being present. This reaction may be of importance in the generation of polysaccharide in embryonic tissue where little or no primer polysaccharide is present.

Leloir and coworkers have discovered an alternate pathway for glycogen biosynthesis. The reaction

$$\text{UDPG} + (\text{Glucosyl})_n \longrightarrow \text{UDP} + (\text{Glucosyl})_{n+1}$$

was first demonstrated in liver (10), and the enzyme was later purified from muscle (11). This enzyme, UDPG glycogen transglucosylase, mediates the donation of a glucose unit in α-(1 $\longrightarrow$ 4) linkage to amylose or glycogen. It cannot cause further branching of the molecule and does not appear capable of degrading it.

<u>Variations in Glycogen Structure</u>

Reports in the literature indicate that structural modifications of glycogen occur to a limited extent. Wolfrom and Thompson found isomaltotriose and nigerose among the acid hydrolysis products of beef liver glycogen (12). This indicates consecutive α-(1 ⟶ 6) linkages occur to a limited extent in adjacent positions in the molecule and that some α-(1 ⟶ 3) linkages are also present. The hydrolysis technique is designed to minimize reversion (23).

The glycogen hydrolysate was fractionated by column chromatography into disaccharide and trisaccharide components. The disaccharide mixture was subjected to acetylation, fractional crystallization, and column chromatography. This procedure yielded isomaltose and maltose octaacetates both of which are predicted from the classical structure of the glycogen molecule. In addition a very small amount (2 mg.) of nigerose octaacetate (3-O-α-D-glucopyranosyl-D-glucopyranose octaacetate) was isolated. This is an extremely small amount considering 92 grams of glycogen were hydrolized.

The trisaccharide mixture was fractionated and subjected to preparative electrophoresis. One component was isolated which showed an optical rotation similar to that reported for isomaltotriose. A molecular weight determination indicated it to be a trisaccharide. A sample of this material was reduced with hydrogen under pressure. It was subsequently partially hydrolized with acid and the hydrolysis products were acetylated. The mixture was subjected to Magnasol-Celite column chromatography. Three components were resolved by this procedure. They were identified as isomaltitol nona acetate, β isomaltose octaacetate, and D-glucitol (sorbitol) hexacetate. The effluent also contained one component identified as D-glucopyranose pentaacetate. The fact that isomaltitol nona acetate was isolated proves that the reducing hexose is linked through its six position to the second monosaccharide. Since isomaltose octaacetate was isolated and the parent compound was a trisaccharide, the second hexose must be linked through its six position to the third sugar in the trisaccharide. This type of fragmentation analysis may be easily compared with the amino acid sequence technique commonly employed in protein structural studies (24).

Methylation of glycogen, followed by hydrolysis of the methylated derivative, is a useful technique in structural determinations. This process should produce a molar proportion of di-O-methyl-D-glucose (from the glucose units located at the branch point) equal to that of 2,3,4,6 tetra-O-methyl-D-glucose if the postulated structure of glycogen is correct. Reports (3,25,26) indicate, however, that the ratio of di-O-methyl-D-glucose is significantly higher than the ratio of the 2,3,4,6 tetra-O-methyl derivative. This has been attributed to incomplete methylation. The di-O-methyl-D-glucose (25-27). This could be a result of either incomplete methylation or demethylation brought about during hydrolysis. Further work will have to be done to ascertain fully the significance of these other derivatives.

Abdel-Ahker and Smith (28) have made use of an unusual technique to study the (1 $\longrightarrow$ 3) linkage postulated in glycogen. Several glycogen specimens were each treated with periodate yielding a polyaldehyde. This was chemically reduced to a polyalcohol and this product subjected to acid hydrolysis. The accepted structure of glycogen demands that terminal non-reducing groups each yield one mole of glycerol while all other residues give erythritol. Glycolaldehyde is split from all residues also. However, in addition to these three compounds, free glucose was found to be a component of the hydrolysis mixture in all samples tested. This is strong evidence in favor of a (1 $\longrightarrow$ 3) linkage, because glucose units with this type of linkage would be inert to attack by periodate. A second treatment of the polyalcohol with periodate still failed to eliminate glucose as a product of hydrolysis. Their data indicate that approximately one out of 120-150 glucose units are joined by a (1 $\longrightarrow$ 3) linkage. Although the possibilities of a (1 $\longrightarrow$ 2) linkage cannot be discounted by this type of evidence, these experiments coupled with the isolation of nigerose octaacetate strongly suggest that some (1 $\longrightarrow$ 3) linkages are indeed present. The presence of (1 $\longrightarrow$ 3) linkages has been proven in amylopectin (29) and is indicated in glycogen from the snail *Helix pomatia* (30).

Peat, Roberts, and Whelan reported (31) the isolation of 4-O-α-D-glucopyranosyl-D-fructose (maltulose) from chromatographic effluents of amylase hydrolyzates of pregnant rabbit liver glycogen (Figure 1). More than 200 mg. of maltulose were obtained from six grams of glycogen 2:3-4:5 diacetone fructose was prepared from a yeast α glucosidase hydrolysate of the isolated maltulose and the original disaccharide also yielded a phenylosazone identical to maltosazone. Authentically synthesized maltulose compared in optical rotation, melting point, iodine consumption, and acid hydrolysis rates with the isolated disaccharide. No evidence for the location of this fructose in the glycogen molecule was given but the fact that glucose is linked to C4 of fructose would indicate that this ketose is in a position other than the terminal non-reducing point in the branches.

Certain discrepancies in the average length of the repeating unit of glycogen have been reported. Bell found that when fasted rabbits were given galactose, they produced glycogen consisting of 18 glucose molecules per repeating unit (32). This was deduced from the percentage of 2,3,4,6 tetra-O-methyl-D-glucose obtained from methylation and hydrolysis. Bell also reported (33) that glycogen from whole tissue of the mussel *Mytilus edulis* contained 18 glucose monomers in the average repeating unit (i.e., $\overline{CL}$ = 18).

Haworth *et al.* (3) obtained rabbit liver glycogen from a commercial source which was found to contain a $\overline{CL}$ of 18 as evidenced by methylation data. Halsall, Hirst, and Jones repeated the work of Haworth, but used periodate oxidation to measure the repeating unit size (34). They used the same specimen of glycogen and obtained a $\overline{CL}$ of 18 also. Further, they examined another commercial sample of rabbit liver glycogen and obtained a $\overline{CL}$ value of 18. All other samples examined had a $\overline{CL}$ of 12. Methylation studies by Bacon *et al.* (35) revealed that the feeding of sucrose to rabbits produces a liver glycogen with a $\overline{CL}$ of 18 while fructose and glucose diets led to the formation of 12 unit glycogen. Rabbits used as sources of commercial glycogen are often fed sucrose to stock the livers with polysaccharide. This may explain why these two samples of glycogen have an abnormal average repeating unit size.

Abdel-Ahker and Smith (8) also have conducted experiments to study the average repeating unit of glycogen. They examined glycogen from 37 sources from many different species. Periodate oxidation was employed to determine repeating unit size. Galactose when administered to horse, rabbits, and guinea pigs, gave glycogen with a $\overline{CL}$ of 11-13.

This is in disagreement with the work of Bell (32). One rabbit liver sample was checked using methylation analysis and gave a value of 12 by this method also. A sample from Mytilus edulis was found to contain a $\overline{CL}$ of 12 in contradiction to the value reported earlier by Bell (33). In none of the thirty control samples, including one from a commercial source, was a glycogen with an average repeating unit of 18 encountered. The authors suggest that since the majority of data favoring 18 unit glycogen has come from methylation studies this may represent a loss of the volatile 2,3,4,6-tetra-O-methyl-D-glucose during fragmentation analysis. However, further work is necessary to determine the effect of feeding sucrose to rabbits on the nature of the glycogen produced. Enzymatic studies (36) for end group assays involving the successive action of salivary amylase and plant R-enzyme indicated that a sample of rabbit liver glycogen had an average chain length of 12.5. A check of this sample by periodate oxidation gave a value of 13.6.

Illingworth and Cori (37) in 1952 described several cases of abnormally high glycogen deposition in the tissues of humans. Later work (38) by this group resolved the deposition disorders into four general types.

Type I An accumulation of glycogen of normal structure in the liver and kidneys, with an associated deficiency of glucose-6-phosphatase. This condition is known as von Gierkes disease.

Type II A generalized storage disease in which all tissues contain large quantities of glycogen of normal structure. As the heart muscles are affected, infants usually die within a few months after birth (15).

No enzyme deficiencies have been reported. There does not appear to be any elevation of phosphorylase or UDPG glycogen transglucosylase in tissues of patients with this type of disorder (39).

Type III Characterized by glycogen with short outer branches. Glycogen accumulation in tissues is again generalized and is due to a specific lack of the enzyme amylo 1—> 6 glucosidase.

Type IV Only one case has been observed to date by Cori's group (37). This polysaccharide had a $\overline{CL}$ of 21 and stained red-purple with iodine. Actually the molecules appeared to resemble amylopectin in physical character. Only liver "glycogen" was analyzed but other clinical evidence indicated the accumulation was general in this particular patient.

Polglase, Brown, and Smith (40) have studied liver and muscle glycogen preparations from normal humans, animals and human subjects with glycogen storage disease and von Gierkes disease. All human glycogens were found to contain two components as evidenced by sedimentation data except for the patient with von Gierkes disease. This glycogen was found to contain only one component. Rat glycogen was found to be composed of one component while rabbit liver glycogen was compsed of two. Most human muscle glycogens were found to be made up of two fractions while rat muscle glycogen was composed of only one.

The authors feel that the absence of the secondary component in the patient with von Gierkes disease may be of significance. It is possible that the enzymatic disorder associated with the condition may be accompanied by a change in the physical nature of the glycogen.

The majority of ultracentrifuge studies tend to indicate that most glycogens are homogenous. Lewis and Smith, however, have reported(41) the unusual fact that all glycogens studied in their laboratory are electrophoretically heterogeneous. Using glass fiber paper and sodium hydroxide as solvent, glycogens were all found to migrate as two distinct components. In all cases the slower components all migrated at the same rate but the faster major components of different glycogens had varying rates of mobility. Heterogeneity was also observed with amylopectin and potato amylose. In addition to the above material many other types of polysaccharides were studied. Some displayed homogeniety while others were heterogeneous by electrophoretic criteria.

MATERIALS AND METHODS

Reagents and Substrates

Rat glycogen was obtained from a mixed male and female population. A lactating Holstein cow and Hereford steers served as sources of bovine glycogen. Various glycogen preparations were also obtained from Leghorn chicks. Highly purified potato starch was a gift from Dr. Jean Burnett. Maltose was obtained from Pfansteihl Chemical Company, further purified by column chromatography on Darco G-60 Celite (42) and recrystallized twice from aqueous ethanol. The purified maltose melted at 118° (43) and had a specific rotation of +130° (44). Galactose C^{14} was purchased from Volk Chemical Company and had a specific activity of 1.38 μc/mg. Other sugars were obtained from Pfansteihl Chemical Company as reagent grade compounds and were used without further purification. Defatted Jack Bean meal was purchased from Sigma Chemical Company. Amberlite IR45 (OH-) and IR120(H+) ion exchange resins were purchased from Rohm and Haas Company. Glucose oxidase (purified) containing catalase, was obtained from the Nutritional Biochemicals Company. Methylphenyl hydrazine was purchased from the Eastman Chemical Company.

Galactose-methyl-phenyl hydrazone was prepared as described by Hirst, Jones, and Wood (45). The white crystals melted at 185-186° (uncorr.) and were optically inactive (43). The presence of glucose or maltose did not interfere with the preparation of this compound (45). Concanavalin A was prepared by the method of Cifonelli and Smith (46).

Qualitative Measurements

Descending chromatography was carried out on Whatman No.1 filter paper for the identification of hydrolysis components of the polysaccharides. Whatman No.3 paper was used for preparative chromatography. Solvent systems employed were butanol-pyridine-water (6:4:3 v/v) for one dimensional chromatograms (47). For two dimensional systems water saturated phenol (46) was used in the first direction with butanol-pyridine-water (6:4:3 v/v) in the second direction. Detection of spots was accomplished using the ammonia silver nitrate method of Trevelyan (49). Radioautography was accomplished using Kodak no screen X-ray film.

Quantitative Measurements

Total phosphorus determinations were performed using King's method (50) or by the Fiske-SubbaRow procedure as outlined in Methods in Enzymology (51). Reducing power was measured by the method of Park and Johnson (52) and total carbohydrate by using the phenol sulfuric acid procedure of DuBois *et al.* (53). Nitrogen was determined utilizing a modified Nesslers reagent (54). Radioactivity measurements were made in a scintillation spectrometer. The samples were dissolved in 0.01 to 0.1 ml H_2O and 5.0 ml of absolute ethanol. Then 10 ml of scintillating fluid were added which was made from 4.0 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene made up to one liter with toluene (55).

Thixatropic gel was obtained from Packard Instrument Company and was used as a 2 1/2% solution in the scintillating fluid to enable counting concentrated sugar solutions.

Optical rotations were determined in a Model D Keston polarimeter unit using a 0.5 dm cell with a Beckman Model D.U. spectrophotometer equipped with a Gilford recording attachment (56). Periodate oxidations were performed according to the method outlined by Abdel-Ahker and Smith (8) but using a sucrose standard, NaOH as a neutralizing agent, and methyl red as indicator (57). Ultracentrifugation of glycogen was performed in a Spinco Model E analytical ultracentrifuge with the assistance of Mr. Harold Swaisgood of the Department of Food Science, Michigan State University.

Maintenance of Chicks

One day old chicks were obtained from the Poultry Science Department of Michigan State University and distributed at random in heated brooders. They had free access to water and feed at all times.

One group was fed a completely synthetic basal diet which is detailed elsewhere (58). The second group received exactly the same ration but with galactose (Pfanstiehl N.F.) substituted for a portion of the glucose so it accounted for 15% of the total diet. The chicks were observed for the onset of toxicity symptoms (about seven days) at which time the entire populations were sacrificed for liver tissue.

EXPERIMENTAL PROCEDURES AND RESULTS

Purification of Glycogens

Extraction and Precipitation. The initial purification step was based on minor modifications of the method outlined by Somogyi (59). It combined the use of low alcohol concentrations to remove impurities which adhere to the glycogen, with the use of acid precipitation to eliminate protein contaminants.

Rat and bovine livers were removed immediately after death of the animals, weighed, cut into small pieces and placed in crushed ice. Chick livers were placed directly in the digestion solution after their excision from anesthetized birds. Digestion was carried out by adding 2 ml. of 50% NaOH per gram of wet liver and heating three hours on a steam cone with occasional stirring during the first hour. Upon cooling slowly to room temperature lipid material congealed on top of a clear liquid.

The viscous liquid was gently poured from underneath the solid layer. This layer was then dispersed and heated in a volume of water about equal to that of the original digest. After adding NaCl, a clear liquid was separated by filtration. One half volume of 95% ethanol was added slowly with stirring to the combined filtrates. The crude glycogen was allowed to flocculate overnight at room temperature.

Without disturbing the precipitate, the supernatant solution was gently siphoned off until a small volume remained above the insoluble material which was removed by centrifugation.

A wash solution was prepared by mixing two volumes of 20% NaOH and one volume of absolute ethanol. The crude polysaccharide was resuspended with wash solution in the centrifuge bottles repeatedly until the liquid was colorless. It was finally washed once with 95% ethanol.

The impure glycogen was dissolved in water. Any insoluble material was removed by centrifugation. The clear liquid was treated with 2N HCl at room temperature until the pH of the solution was approximately 2-3 as determined with pH paper. The fine, highly dispersed precipitate which formed was flocculated by the addition of 50 ml. of 95% ethanol for every 100 ml. of solution. It was found at this point that centrifugation was a more efficient means of removal of this insoluble material than filtration. Additional 95% ethanol was added to the centrifugate to raise the alcohol concentration to approximately 45% which was sufficient to cause complete precipitation of the glycogen in the acid medium. The precipitated material was allowed to settle our overnight at 2^{o} and was then collected by centrifugation. It was washed twice with 45% ethanol, twice with 95% ethanol, once with absolute ethanol, once with ether and dried *in vacuo* at room temperature.

The white powder was dissolved in water and dialized against slowly running distilled water at 2^{o} for 6 to 7 hours (about 18 to 20 liters). The glycogen was reprecipitated by the addition of an equal volume of absolute ethanol and washed once with 95% ethanol, twice with absolute ethanol, and finally with ether. Glycogen at this stage of purity will henceforth be called Preparation I (Table I).

Precipitation with Concanavalin A. Concanavalin A is a globulin isolated from Jack bean which shows a narrow range of specificity of a

Table I. Analytical Data for Preparation I Glycogens

Sample	Male Rat	Female Rat	Cow	Basal Chicks	Galactose Toxic Chicks
$[\alpha]_D$(c,0.5 water)	+199°	—	+196°	+193°	+196°
Reducing Power $\frac{\mu g.\ Glu.}{mg.\ Glycogen}$	0.16	—	0.13	—	0.11
Total Phosphorous $\frac{\mu gP}{mg.\ Glycogen}$	0	0	0	<.2	—

precipitin complex formation with polysaccharides (46). Tables II and III list polysaccharides which have been tested by Cifonelli et al. (60, 61) and by the author. In addition the table lists the presence or absence of a visible precipitin reaction based on a turbidimetric method (46).

Concanavalin A shows a high degree of specificity towards glycogens of various sources. It has been suggested that D-glucose polysaccharides which are positive in their reaction with Concanavalin A should be classified as true glycogens.

Two grams of each preparation I glycogen were dissolved in 200 ml. of 2% NaCl and 400 ml. of Concanavalin A preparation were added. A white precipitate formed immediately. This amount of Concanavalin A was found to be in excess since further precipitation could be caused by adding more glycogen to the supernatant solution from the original precipitation reaction. The precipitate was allowed to flocculate one hour at room temperature and was centrifuged. The pellet was thoroughly dispersed in 100 ml. of 2% NaCl and recentrifuged. To the pellet was added 45 ml. of water and 8 drops of 20% KOH. It was stirred until all of the complex was in solution. Finally it was heated three minutes in a boiling water bath to help denature protein and cooled to 0^{o}. Four volumes of ice cold 10% TCA were added to the solution and the resulting precipitate allowed to settle at 0^{o} for 2 hours. It was then centrifuged and the precipitate washed once with 60 ml. of 5% TCA. The centrifugate and wash were combined and glycogen precipitated by addition of 275 ml. of ice cold 95% ethanol. It was washed once with 50% ethanol, once with 95% ethanol(overnight at 2^{o}) and finally once with absolute ethanol.

Table II. Specificity of Concanavalin A Reaction with Various Polysaccharides. (Data from work of Cifonelli *et al.* (60,61))

Polysaccharide	Source	Reaction with Concanavalin A	Known or presumed to contain Galactose
1 Glycogen	37 sources	+	-
2 Glycogen limit dextrin from β amyloysis	liver	+	-
3 Heparin	beef lung	+	-
4 Mannan	yeast	+	-
5 Starch	potato, corn	-	-
6 Amylopectin	potato, corn	-	-
7 Amylose	potato, corn	-	-
8 Dextran	*L. mesenteroides*	-	-
9 Chondroitin sulfate	unspecified	-	-
10 Hyaluronic acid	unspecified	-	-
11 Galactogen	beef lung	-	+
12 Starch after β amyloysis	corn	-	-
13 Glycogen after α amylolysis	liver	-	-

Table III. Specificity of Concanavlin A Reaction with Various Polysaccharides (Data from present work).

Polysaccharide	Source	Reaction with Concanavalin A	Known or presumed to contain Galactose
Glycogen	cow	+	-
Glycogen	basal chick	+	-
Glycogen	galactose toxic chick	+	+
Glycogen	rat	+	-
Starch		-	-

Acetylation Extraction and Deacetylation. The possible merit of acetylating glycogen for the removal of proteinaceous and lipid material has been reported and the data supports this conclusion (60). For the purpose of insuring complete removal of protein from the Concanavalin A step and for the further purification of the glycogen, acetylation, washing and deacetylation were carried out on these preparations.

To the Concanavalin A positive polysaccharides, still moist with alcohol, were added 50 ml. of reagent grade pyridine and the glycogen worked until in solution. Fifty ml. of redistilled acetic anhydride (BP 116 - 117^{o}) were added slowly with stirring but with no external cooling. The reaction vessels were stoppered and allowed to stand 24 hours at room temperature. The solutions were then poured slowly with vigorous stirring into an ice slush mixture. The white precipitates were filtered with suction. They were washed several times with water and refiltered each time. A final wash with 5% ethanol used before drying was at 80^{o} in vacuo for 3 hours.

The deacetylation process was begun in acetone solution to which was added one equal volume of 20% KOH. The two layered system was heated gently ten to fifteen minutes with stirring. At completion of the reaction the lower aqueous layer, containing the regenerated glycogen assumed an opalescent appearance. The layers were separated and the aqueous layer was acidified slightly by adding cold glacial acetic acid slowly to the chilled solution. Precipitation of glycogen occurred subsequently when two volumes of absolute ethanol were added. The glycogen was reprecipitated twice from water with two volumes of absolute ethanol. Before the second reprecipitation each sample was

filtered through a bacterial filter to remove traces of lint and insoluble material. The glycogens were washed successively as described for preparation I glycogens then dried at room temperature in vacuo. The yields of these preparation II glycogens from preparation I material averaged about 60% by weight (Table IV).

Removal of Galactose C^{14} during Purification of Glycogen. Steer liver was divided into two 86 gram batches; 5.0 g. of galactose was added to one and 5.0 g. of galactose + 5.5 mg. galactose-1-C^{14} was added to the other. The sample without radioactive material served as background and was included in counting standards. The specific activity of the galactose was 605 dpm/μmole after dilution. Each sample was digested in alkali and lipid material removed as previously described. It was found that 81% of the total disintegrations added initially were recovered in the liquids immediately prior to the first alcohol precipitation. Fractionation of both glycogen samples was continued up to the point where the glycogens were subjected to dialysis in the previously described purification.

A portion of each sample was then dissolved in water. Determinations of total carbohydrate, reducing power and radioactivity were made. The glycogen was hydrolyzed prior to counting where it was necessary to use a thixatropic gel as described previously in order to obtain a complete suspension of the hydrolyzate at the concentration employed.

The data in Table V show that this portion of the purification process is completely adequate to remove all detectable free galactose

Table IV. Analytical Data for Preparation II Glycogens.

Sample	Male Rat	Female Rat	Cow	Basal Chicks	Galactose Toxic Chicks
$[\alpha]_D$(c,0.5 water)	+194^{o}	+194^{o}	+196^{o}	+191^{o}	+193^{o}
Reducing power $\frac{\mu g \text{ glucose}}{mg \text{ glycogen}}$	0.56	0.091	0.11	0.15	0.08
Total Phosphorous $\frac{\mu g \text{ P}}{mg \text{ glycogen}}$	0	0	<0.1	<0.1	<0.1
Total Nitrogen $\frac{\mu g \text{ N}}{mg \text{ glycogen}}$	—	—	<0.1	—	<0.1

Table V. Removal of Free Galactose from Steer Glycogen During Purification

Sample	Total carbohydrate mg/ml	Reducing power mg/ml	mg counted	CPM	dpm/mg Above background
Radioactive	200	0.002	20	15.8	0
Non-radioactive	200	-	20	15.9	-

even when an amount greater than 5% of the total wet weight of the tissue was added exogenously. It should be noted that the efficiency of the counting was 57% in this experiment. With a specific activity of 605 dpm/µmole, 1 µg of galactose per 2.0 mg. of glycogen would result in about 20 counts per minute above background whereas the glycogen fractionated in the presence of radioactive galactose had no detectable counts. Since the various glycogen preparations were all subjected to further exhaustive purification, it is inconceivable that the galactose found associated with the glycogen could result from free galactose or a low molecular weight contaminant.

Physical Characteristics of Glycogen

Homogeneity. In order to study the possibility that two separate molecular species may occur in galactose toxic chick glycogen, a sample (preparation II) was subjected to ultracentrifugal analysis. A 1% solution in phosphate buffer at pH 5.6, ionic strength 0.1, was sedimented at 12,590 rpm at 20° in a Spinco Model E analytical ultracentrifuge. Photographs were taken at various intervals during a forty minute run. At no time did there appear to be a second major component (40) detectable under the experimental conditions (Figure 3).

There does not appear to be any heavy tailing in the sample, which would indicate impurities (15). The sedimentation coefficient was calculated according to the method outlined by Schachman (62) from photographic enlargements of the 4, 12, 20 and 32 minute pictures. The S_{20_w} was found to be 81.0 x 10^{-13} sec. which is in excellent agreement with values reported elsewhere (11,63) for liver glycogen prepared by

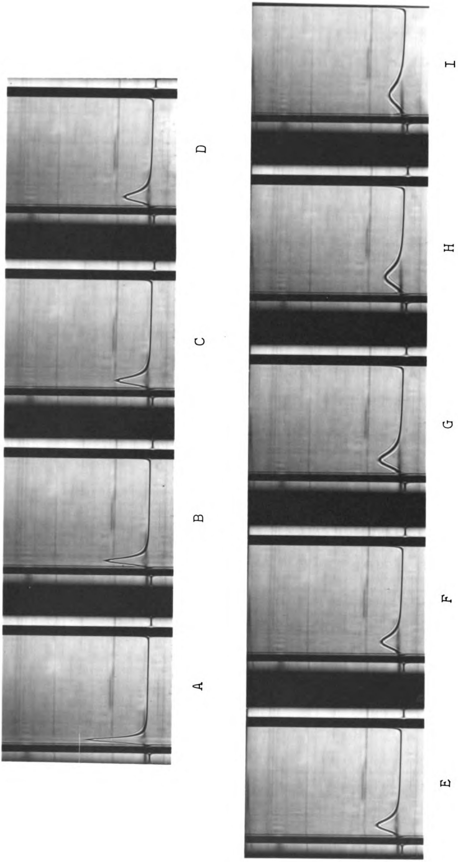
Figure 3.

Photographs of sedimentation patterns of glycogen from galactose toxic chicks taken at various time intervals.

The times given are minutes after attaining 12,590 RPM.

A, 0; B, 4; C, 8; D, 12; E, 16; F, 20; G, 24; H, 32; I, 40.

(For details see text)


A
B
C
D
E
F
G
H
I

alkali extraction. This represents a molecular weight of approximately 5×10^6.

Average Chain Length. These experiments were carried out in order to determine what effect a galactose diet might have on the size of the repeating unit of chick liver glycogen. This could reflect alterations of carbohydrate metabolism due to galactose ingestion.

Samples of glycogen from chicks fed the basal diet, galactose diet, rats (50% female and 50% male by weight) and cow were dried at 110° for two hours. Sucrose, which served as a standard, was treated similarly. Carbon doxide free sodium hydroxide solution was prepared and standardized against analytically pure potassium acid phthalate. Accurately weighed samples of all glycogens were dissolved in water and 20 ml. of 0.5 N sodium metaperiodate were added to each flask before making to a final volume of 100 ml. with water. Samples and standards were quickly cooled and kept in the dark at 5°. Twenty milliliter aliquots of the sucrose solution were removed at intervals (Figure 4), freed of excess periodate with 0.5 ml. of ethylene glycol, and the formic acid titrated.

Blank determinations were carried out as follows:

1. An accurately weighed portion of sucrose (about 16 mg.) was dissolved in 16 ml. of water.

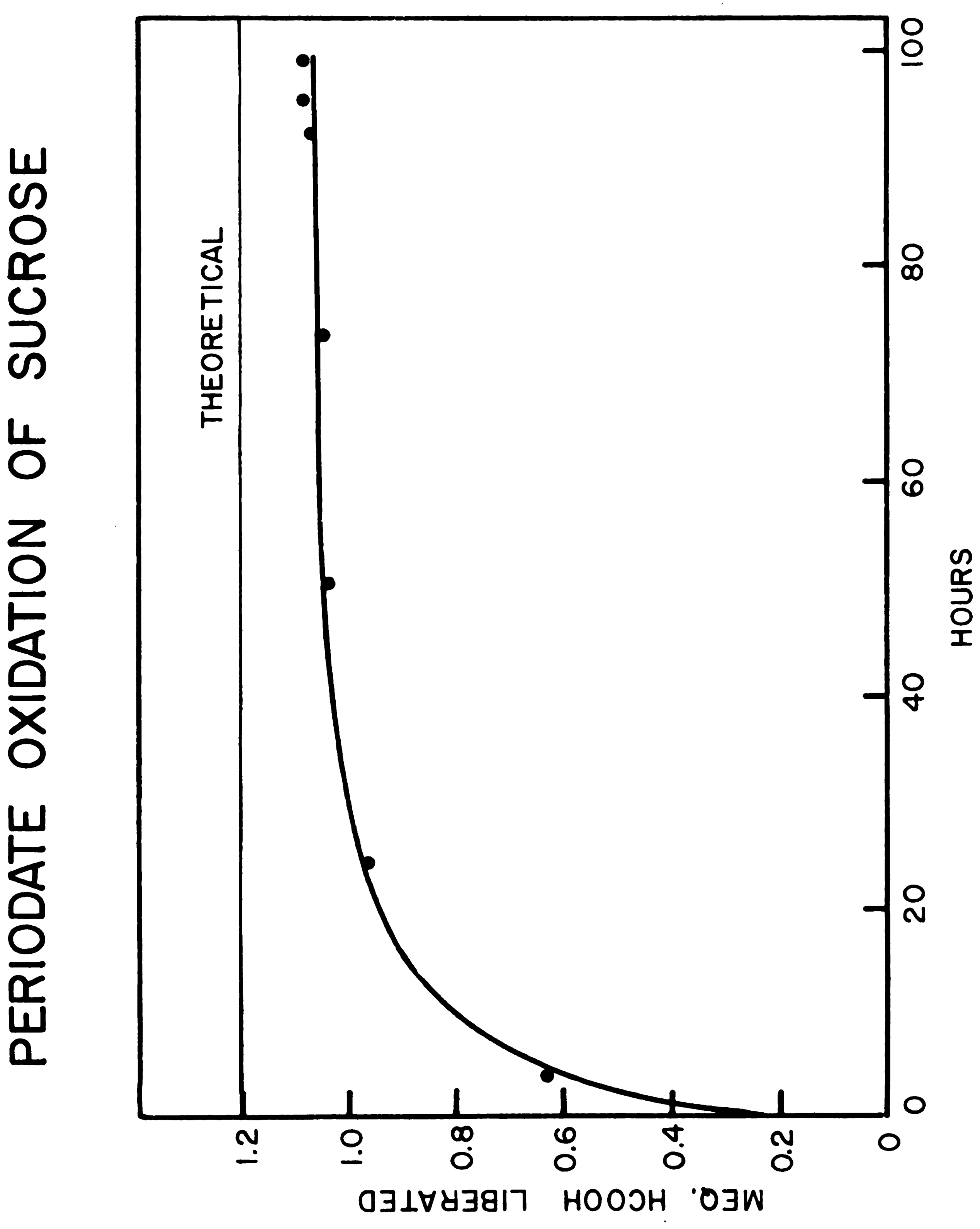
2. Five tenths of a ml. of ethylene glycol and 4 ml. of 0.5 N $NaIO_4$ were added.

3. After standing 15 minutes in the dark at room temperature the samples were titrated.

Figure 4.

Periodate Oxidation of Sucrose

(For Details see Text)


PERIODATE OXIDATION OF SUCROSE
THEORETICAL
MEQ. HCOOH LIBERATED
0
0.2
0.4
0.6
0.8
1.0
1.2
HOURS
0
20
40
60
80
100

As can be seen in Figure 4 the oxidation of sucrose was essentially complete at 92 hours. This represents 91% of theoretical on the basis of sucrose added. Therefore, it was assumed that the glycogen was underoxidized to the same extent and a correction was made for the amount of formic acid produced in calculating the average chain length of the polysaccharides (57).

Fifteen ml. aliquots of aqueous glycogen solutions were titrated at 92, 95 and 99 hours after the addition of periodate reagent. The formic acid yield remained constant over this period. In separate experiments glycogen samples were titrated to check the presence of base titratable material in the absence of ethylene glycol and sodium metaperiodate. These were negative. Calculations of average chain length were made from the average of values obtained after 90 hours incubation. The data in Table VI indicate that the average length of the repeating unit of all glycogen samples examined is the same. There does not appear to be any formation of an 18 unit glycogen from feeding chicks a 15% galactose diet. This is in agreement with the data of Abdel-Ahker and Smith (8) with regard to the effect of galactose ingestion on glycogen structure in animals.

Evidence for Galactose in Glycogen from Galactose Toxic Chicks

Chromatographic. In the following experiments it was assumed that a hydrolyzate of glycogen would produce only a glucose spot when subjected to chromatography. In order to examine the molecule for components which might be present in small amounts, it was considered necessary to

Table VI. Periodate Oxidation of Glycogens

Sample	Total mg. Glycogen Oxidized	Total milli- moles glucose per sample mg/162.4	Millimoles formic acid produced*	Average Length of Repeating Unit
Bovine	79.5	0.490	.0396	12.3
Rat	51.8	0.320	.0256	12.5
Chicks Basal	81.2	0.500	.0396	12.6
Galactose Toxic Chicks	79.1	0.486	.0380	12.8

*Millimoles of HCOOH produced corrected for incomplete oxidation of the sucrose standard.

selectively remove the glucose before chromatography to eliminate a swamping effect of this hexose. Conditions employed for hydrolysis of compounds of this type are such that artifacts of hydrolysis or reversion products are frequently produced. For this reason it was necessary that polysaccharides and oligosaccharides of similar biological origin and chemical composition be used for controls. Throughout the experimental procedures control polysaccharides received exactly the same treatment as the experimental sample.

The various glycogen preparations, maltose and potato starch, were hydrolyzed by dissolving 10 mg. of each in 1.0 ml. of 4 N H_2SO_4 and heating 30 minutes in a boiling water bath. After cooling, the solutions were passed slowly through individual columns (0.6 x 12 cm.) of Amberlite IR45(OH^-) and the monosaccharides were eluted from the columns by washing with water. The removal of sulfate ion was complete as evidenced by failure of the effluents to form a precipitate in the presence of 0.3 N $Ba(OH)_2$. The eluates, about 10 ml. pH 4.5, were evaporated to dryness. Residual material was dissolved then transferred to the main compartment of Warburg flasks with the aid of a micropipette. Three 0.3 ml. fractions of 0.4 M Na acetate-cacodylic acid buffer at pH 5.6 were added to aid complete transfer of carbohydrate to the Warburg vessel. The large excess of glucose in the hydrolysate was removed by glucose oxidase. This was prepared by dissolving 4.0 mg. of the enzyme preparation in one ml. of .05 M Na acetate-cacodylic acid buffer pH 5.6. An aliquot (0.15 ml.) of this enzyme solution was added to the sidearm and the flasks equilibrated with shaking for 10 to 15 minutes at 30°. The components were mixed and when oxygen uptake ceased the reaction was discontinued. The solutions were then removed from the flasks and the flasks rinsed several times with water. To remove electrolytes the combined reaction mixture and rinsings were put through individual columns (0.6 x 10 cm.) of Amberlite IR120(H^+) and Amberlite IR45(OH^-) prepared by constructing equal alternate layers of the two resins in the same column. Following thorough washing of the columns with water the effluents were evaporated to dryness and then dissolved in 0.05 ml. water and applied to chromatograms or stored in the deep freeze.

In a series of trial experiments with bovine glycogen this hydrolysis procedure was followed using added radioactive galactose. About 80% of the radioactive galactose could be recovered for chromatography. This recovery seemed reasonable considering the manipulations involved.

Glycogen from the various sources, starch and purified maltose, were hydrolyzed and prepared for chromatography as described above. Four representative chromatograms are photographed in Figure 5. Hydrolyzates from all of these specimens show an identical chromatographic pattern with the exception of the hydrolyzate of glycogen from galactose toxic chicks. This hydrolyzate shows one extra major component. Identification of the common components is as follows: The dark spot nearest to the origin is gluconate. This was deduced from a comparison of mobility of authentic gluconic acid in both solvent systems independently. The gluconate is present as a result of incomplete removal during deionization of the enzymically treated hydrolyzates. The spot immediately to the left and above gluconate is probably isomaltose as judged by its mobility relative to glucose in both solvent

Figure 5

Photographs of chromatograms of hydrolyzates of various polysaccharides.

Direction of solvent flow is indicated by arrows.

Spots identified in each chromatogram from left to right

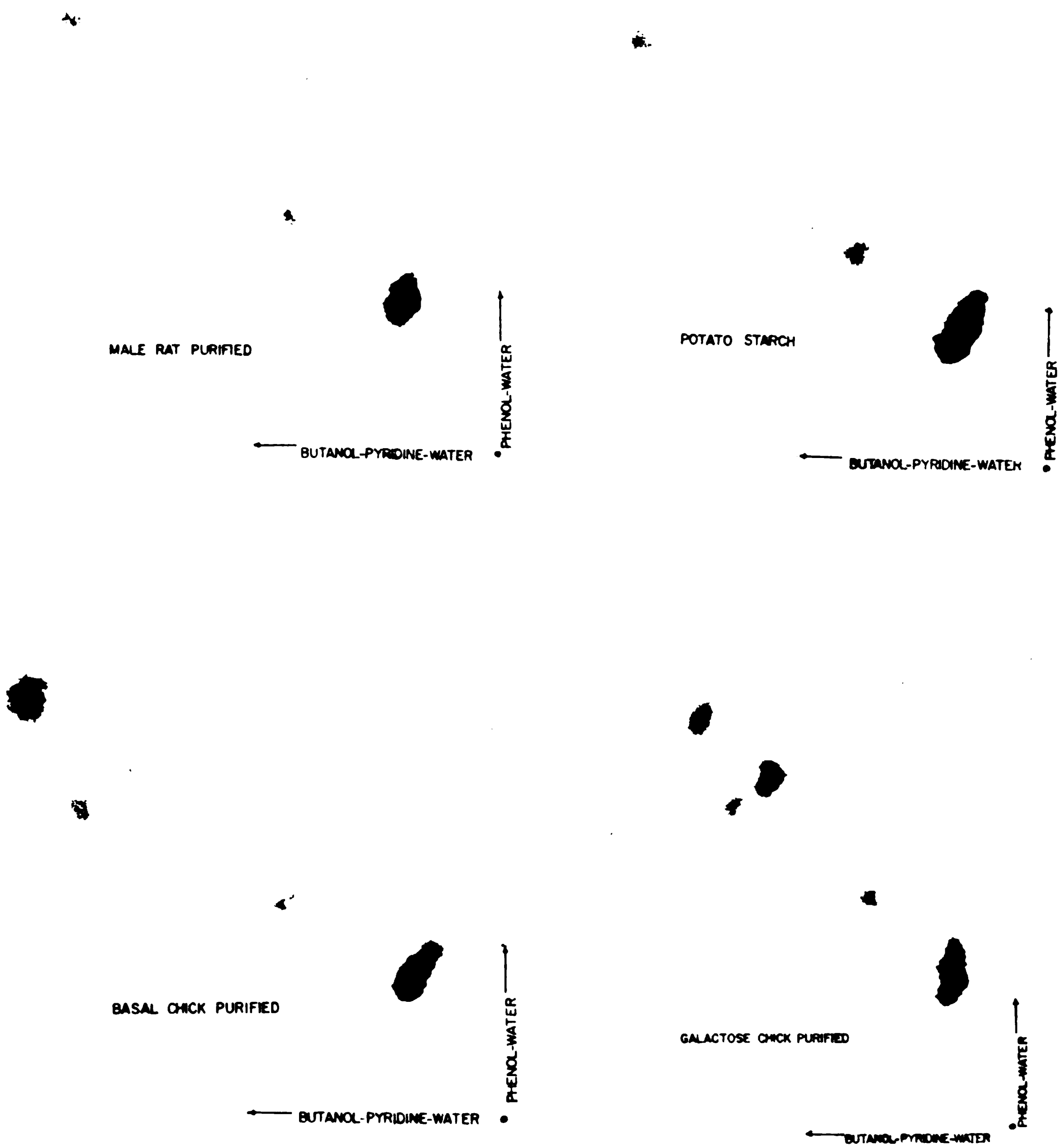
Male Rat: Fructose, Isomaltose and Gluconic Acid.

Potato Starch: Fructose, Glucose, Isomaltose, and Gluconic Acid.

Basal Chick: Fructose, Glucose, Isomaltose, and Gluconic Acid.

Galactose Toxic Chick: Fructose, Glucose, Galactose, Isomaltose, and Gluconic Acid.

(For details see text)


MALE RAT PURIFIED
PHENOL-WATER
BUTANOL-PYRIDINE-WATER
POTATO STARCH
PHENOL-WATER
BUTANOL-PYRIDINE-WATER
BASAL CHICK PURIFIED
PHENOL-WATER
BUTANOL-PYRIDINE-WATER
GALACTOSE CHICK PURIFIED
PHENOL-WATER
BUTANOL-PYRIDINE-WATER

systems (47,64). Further when this compound is eluted and hydrolized in acid, glucose is the only sugar detectable in the hydrolyzate. Glucose is to the left and above isomaltose. In some cases the enzymatic oxidation effects complete removal of glucose from the hydrolyzates for example in the male rat glycogen sample. In the photograph of the hydrolyzate of the glycogen from galactose toxic chicks, galactose appears to the right and above glucose. The fastest moving component in all chromatograms is fructose. The identity of these last three sugars mentioned is based upon comparison of their mobilities relative to glucose in the two solvent systems and with authentic materials. The identity of this extra component as galactose was further confirmed by its co-chromatography with authentic galactose C^{14}.

The presence of glucose is naturally expected. Isomaltose is a fragmentation product resulting from the hydrolysis of starch and glycogen. Its presence in the maltose hydrolyzate is probably due to acid reversion (65). The fructose arises most probably during hydrolysis by an acid catalized isomerization or during neutralization or deionization by a base catalysis (66).

Glycogen from chicks fed the basal diet shows a very faint galactose spot. The fact that chicks tend to incorporate this sugar even on a basal diet may be related to the extreme sensitivity of chicks to galactose.

<u>Galactose in Components Separated from Glycogen by Factional Precipitation.</u> To define further the relationship between galactose and the glycogen molecule, fractional precipitation of glycogen was performed. Glycogen from chicks fed galactose was isolated and purified as

described above for preparation II. Fifty mg. of this preparation were dissolved in 5.0 ml. of water and absolute ethanol was added slowly until the first well defined precipitate was formed. The precipitated polysaccharide was removed by centrifugation and the supernatant liquid transferred to a second vessel. To this supernatant more alcohol was added until a second well defined precipitate formed which was separated as before. This process was repeated until three fractions were obtained (see Table 7). The separate fractions were washed once in 4.0 ml. of absolute ethanol, once with ether and were dried *in vacuo*. All three fractions showed the typical iodine color. The supernatant from the third fraction was treated with additional alcohol but no further precipitation occurred. It was therefore concentrated and dried without washing. Ten mg. each of fractions 1, 2, and 3 and the entire residue from the supernatant (fraction 4) were hydrolized and treated in the usual manner for removal of glucose. As evidenced by chromatography, the galactose was present in all components of the glycogen (Figure 6). Lanes 1, 2, and 3 are from hydrolyzates of fractions 1, 2, and 3 respectively. Lane 4 contains material from the final supernatant. There is no single fraction which contains all of the galactose. A low molecular weight oligosaccharide as a contaminant should be ruled out as it would accumulate primarily in fraction four. This experiment indicates very strongly that the galactose is associated with a polysaccharide.

The Isolation of a Crystalline Derivative of Galactose from Glycogen. Five grams of glycogen from chicks fed galactose (preparation I) was hydrolized in 2 N H_2SO_4 (100 ml.) for 90 minutes in a boiling water

Table VII. Fractional Precipitation of Glycogen.

Fraction No.	Total ml. EtOH added	Wt. Fraction (mg.)	Iodine color
1	3.2	11.1	Red Brown
2	3.3	16.2	Red Brown
3	3.6	14.3	Red Brown
4	4.9	—	—

Figure 6

Photograph of chromatogram of hydrolyzates of fractions obtained from fractional precipitation of glycogen from galactose toxic chicks.

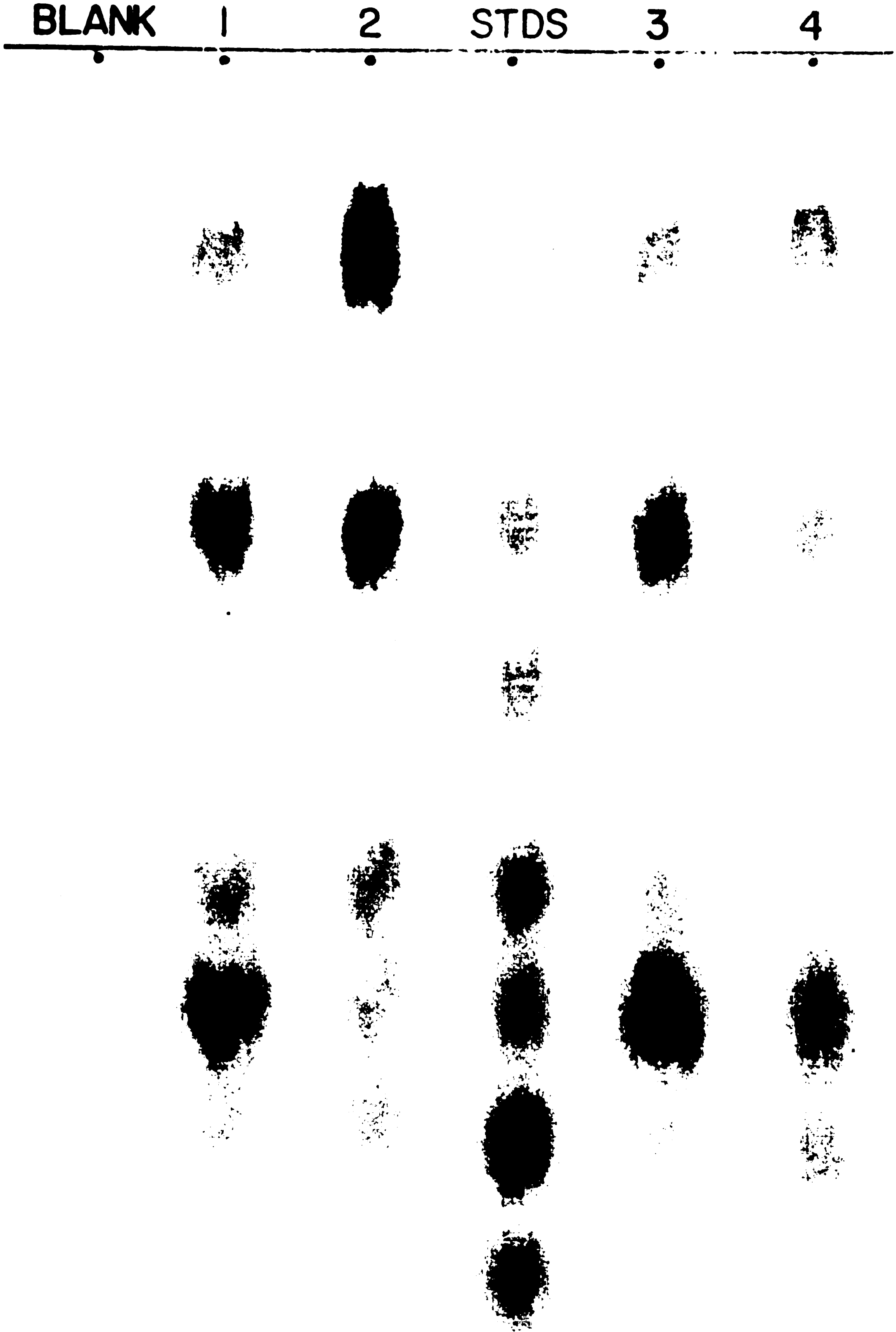
Lane Nos. 1, 2, 3, and 4 are material from glycogen fractions 1, 2, 3, and 4 respectively.

Standards according to increasing mobility: Lactose, Maltose, Galactose, Glucose, Mannose, and Xylose.

Solvent System: Butanol:Pyridine:Water (6:4:3 v/v) descending; 24 hours.

(For details see text)

FRACTIONAL PRECIPITATION



bath. The hydrolyzate was cooled and neutralized (pH 4.5) by the slow addition of solid $Ba(OH)_2$ then a solution of 0.3 N $Ba(OH)_2$. The $BaSO_4$ was removed by centrifugation and the precipitate was washed twice with water. The washings and original supernatant liquid were combined, filtered, and evaporated to a syrup under reduced pressure. The syrup was diluted with 250 ml. of 0.4 M acetate buffer pH 5.6 and 25 mg. of glucose oxidase preparation were added to oxidize the glucose. The reaction mixture was stirred mechanically and change of reducing power was used to follow the course of the reaction which required about 16 hours at room temperature. The reaction mixture was deionized by passing it through a column (42 x 2.1 cm.) of alternate layers of Amberlite IR120(H^+) and IR 45(OH^-). The column was washed with water and the neutral sugars were concentrated. This deionizing and washing process was repeated. The effluent was concentrated to a syrup and diluted in 30 ml. of water and frozen. Half of the material was concentrated and applied evenly on a line on two sheets of Whatman No. 3 paper (46 x 57 cm.). The chromatograms were developed in butanol, pyridine and water (6:4:3 v/v) for 24 hours and then air dried. Marker strips were cut from the edges and center of each sheet, the galactose band was located, excised, and eluted with water. An estimate of total carbohydrate (53) indicated that approximately 6.2 mg. was present (not all galactose). The liquid was concentrated to a syrup and then 0.3 ml. H_2O added. Three tenths milliliter of freshly prepared methyl phenyl hydrazine reagent (45) was added and the mixture was held overnight at 33°. A few crystals formed during this time. The reaction mixture was placed in an ice chest for a few hours and more crystallization occurred. The crystals were collected by centrifugation, washed several times with ice cold 45% ethanol and dissolved in hot 45% ethanol. Insoluble material was removed by centrifugation and the supernatant liquid containing the dissolved derivative was transferred to another vessel for recrystallization. The crystals were washed in ice cold 45% ethanol, ice cold absolute ethanol and dried in a dessicator in vacuo.

Authentic material melted at 185 - 186°; material obtained from the glycogen at 185 - 186°. The mixed melting point was 184 - 185° (all uncorr.).

To further identify the galactose, a small quantity of the crystalline methyl phenyl hydrazone isolated from the glycogen hydrolyzate (e.g. 0.5 mg.) was dissolved in 0.2 ml. of hot water. One drop of benzaldehyde was added and the heating continued for a few minutes. The mixture was cooled and a white oil formed in the aqueous (upper) layer. The aqueous layer was separated from the excess benzaldehyde. Benzyl methyl phenyl hydrazone was extracted with ether and a portion of water solution was placed on chromatograms. The same procedure was carried out using authentic galactose methyl phenyl hydrazone. Galactose was the only sugar present in both samples as judged by chromatography and co-chromatography with authentic galactose in butanol, pyridine and water (6:4:3 v/v).

In a second experiment 5.0 g. of glycogen were hydrolized and treated as above except that during the experiment 0.001 M NaF was present in the incubation of the hydrolyzate with glucose oxidase to eliminate the possibility that the galactose was a result of bacterial contamination. The derivative was recrystallized three times from 45% ethanol. Five and one-half mg. of crystalline product were recovered which melted at 185 - 186° (uncorr.).

DISCUSSION

It does not appear from the data presented, that the galactose isolated as a crystalline derivative from glycogen from chicks fed toxic amounts of galactose could be the result of incomplete purification. The absence of chemical reducing activity in the glycogen eliminates consideration of a monosaccharide impurity. This is further substantiated by an experiment in which free radioactive galactose was removed during the first few steps employed in purification of the glycogen. The total phosphorus values are so low that galactose esterified with phosphorus is ruled out. Nitrogen data eliminate glycoprotein impurities from consideration.

Examination of the properties of the glycogen as a polymer indicates quite clearly that the galactose is chemically associated with the glycogen. Precipitation of glycogen with Concanavalin A or acetylation, extraction and deacetylation do not remove the galactose (Figure 7). Since it is known that free sugars do not react with Concanavalin A, the galactose must be a part of a polysaccharide. Furthermore, as all glycogens react to the exclusion of many other polysaccharides, the galactose is very probably a component of the glycogen.

Fractional precipitation of galactose glycogen shows that galactose is associated with the three fractions obtained and not solely with any one fraction. Failure of the residual material to contain unexpected amounts of galactose points out that a low molecular weight oligosaccharide is not responsible for the appearance of the galactose

Figure 7

Photograph of chromatogram of hydrolyzates of various polysaccharides.

Lane 1 Potato Starch

Lane 2 Glycogen from Galactose Toxic Chick Preparation I

Lane 3 Glycogen from Galactose Toxic Chick Preparation II

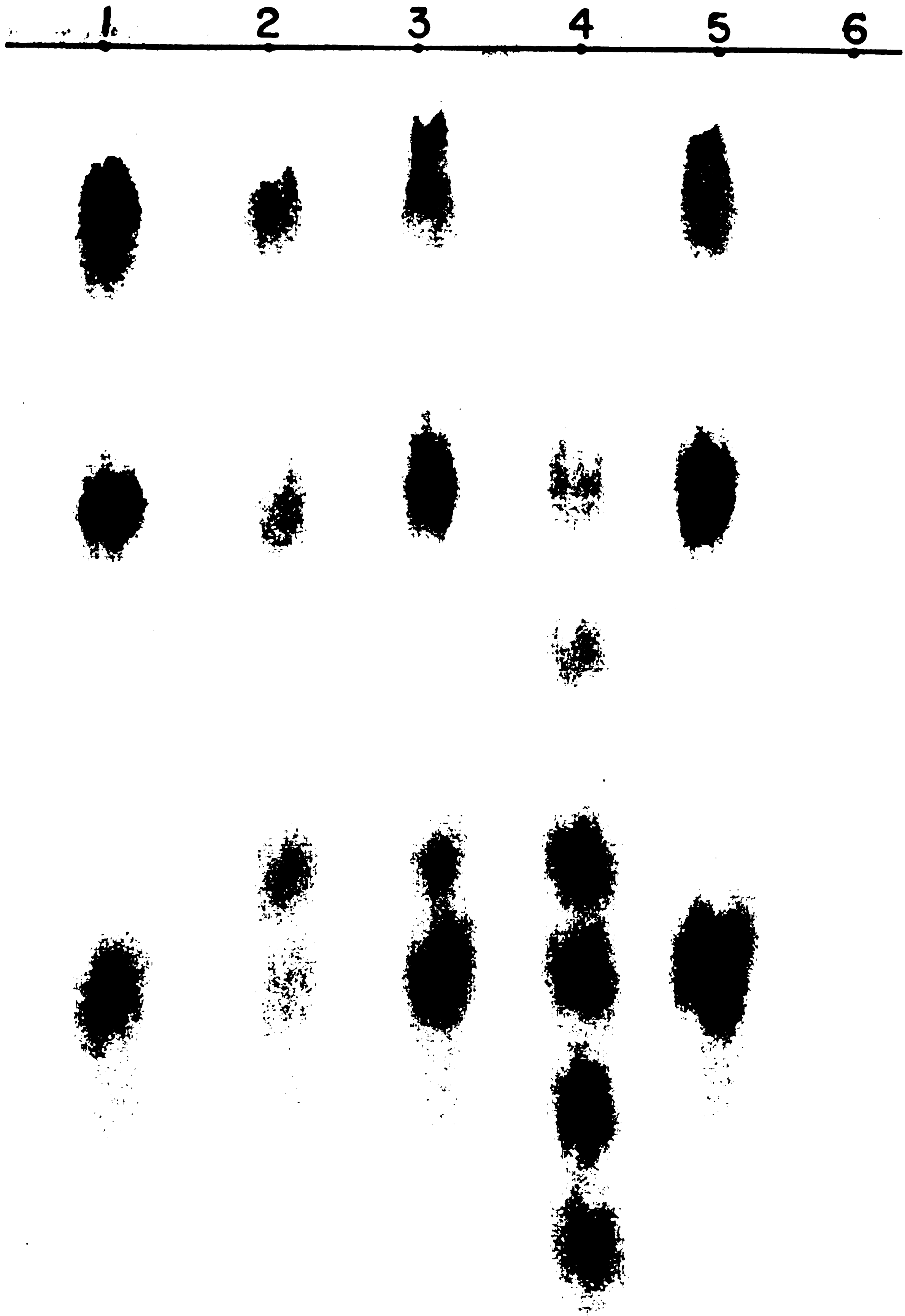
Lane 4 Standards from top to bottom: Lactose, Maltose, Galactose, Glucose, Mannose and Xylose.

Lane 5 Maltose (hydrolyzate)

Lane 6 Reagent Blank

Solvent system: Butanol:Pyridine: water (6:4:3 v/v) descending; 24 hours.

(For details see text)


1
2
3
4
5
6

in the hydrolyzates. Chromatographic evidence indicates the amount of galactose is approximately the same in the glycogen at various stages of purification.

The ultracentrifuge patterns show that the glycogen does not tend to separate into two components. There does not appear to be any tailing which would suggest the presence of high molecular weight impurities or a small second polysaccharide component.

Hydrolysis procedures do not seem to be responsible for the galactose. It was not a component of the hydrolyzates of cow or rat glycogens. Potato starch and maltose were also tested with negative results. It has been suggested (65) that proper control data for reversion products and artifacts occurring during hydrolysis and structural analysis is best obtained if di and oligo saccharides containing the main polymeric linkage of the polysaccharide are employed rather than the monosaccharide itself.

Dursch and Reithel concerned with the mechanism of the glucose-galactose interconversion in biological systems hydrolized glucose-4-phosphate at acid and neutral pH and also with enzymes, to test the possibility that the C-O bond at C-4 of glucose might be cleaved resulting in a Walden inversion forming galactose. No measurable amounts of galactose were obtained as evidenced by paper chromatography (67). Although the same linkage arrangement is present in glycogen, the possibility for galactose formation during hydrolysis was eliminated in the present experiments by the use of appropriate controls.

Other natural carbohydrate polymers contain a small amount of a second sugar. Although the occurrence of these trace components is well documented the purpose they serve and the method by which they are incorporated into polymers is at present not clear. Xylans from land plants are formed from β-(1 ⟶ 4) linked xylopyranose units. The majority of these xylans, however, contain small amounts of L-arabanofuranose and D-glucopyranosyl uronic acid attached as single unit side chains (68).

Mannans, from ivory nut, contain primarily β-(1 ⟶ 4) linked mannopyranose units with two types of non-reducing end groups. Some chains terminate with D-mannose while others contain terminal non-reducing groups of D-galactose (69). It is not known whether two molecular species are present, one containing D-mannose terminal groups and the other containing D-galactose units, or whether the D-galactose is present as single unit side chains. Some classes of mannans are reported to contain D-glucose solely at the terminal reducing end of the molecule (68).

Inulin, a β-(2 ⟶ 1) fructofuranoside polymer, found in Jerusalem artichoke, has been shown to contain glucose in such proportion as to probably be present as one unit per inulin molecule. Hydrolytic enzymes can cause the degradation of this inulin to fructose and small amounts of sucrose (70). Although sucrose is the terminal group, glucose may be found elsewhere in the molecule (71).

The levans (β-(2 ⟶ 6) linked fructofuranoside polymers) of various plant species have also been found to contain 0.4 to 5.2% glucose as evidenced by reactivity of acid hydrolyzates with glucose oxidase (72). The glucose content varies proportionally with the molecular weight of these substances. One inulin preparation was found to have a constant glucose content before and after acetylation and deacetylation of the polysaccharide. Levan preparations retained their glucose after several reprecipitations with ethanol. If the glucose were a contaminant, pruification should tend to lower the glucose content.

Hehre has studied a dextran, (β-(1 ⟶ 6) linked glucopyranose units) produced by a Streptococcus strain which contains 0.27 to 0.37% fructose (73). No actively reducing terminal glucose was present in the molecule. In its place was found a fructofuranoside grouping which was susceptible to the action of invertase. It is believed that the enzyme responsible for synthesis of this dextran utilizes sucrose as an initial acceptor molecule in the synthesis of the polymer to a higher degree than the usual dextran sucrase enzyme. A large number of dextrans examined by Jeanes _et al._ (74) have been found to contain only about 0.02% fructose which is considerably less than that reported for the dextran studies by Hehre.

Laminarin, the food reserve of brown seaweed, is a polymer of β-(1 ⟶ 3) and β-(1 ⟶ 6) linked glucose units (75). Structural work indicates that two main chains terminate by one being attached to C-1 of mannitol while the second chain is attached to C-6 (76). Laminarin molecules which do not contain mannitol have a degree of polymerization approximately equal to one half of those containing mannitol. Small amounts of mannose have also been demonstrated in laminarin (77).

Although these examples are drawn from the plant kingdom, the unique feature of these compounds is the presence of a major and a minor component. It does not seem that these trace components are the result of a biochemical accident due to an over abundance of a second sugar in the cell at the time of synthesis as they are widespread in nature.

The relationship between variations in structure of glycogen and other homopolymers and the biochemical implications of these alterations must await further study.

SUMMARY

Small amounts of galactose have been demonstrated in highly purified preparations of galactose toxic chick liver glycogen. Fractional precipitation of the glycogen fails to remove the galactose. It is not removed by specific precipitation with concanavalin A or by acetylation and deacetylation of the glycogen. Fractionation of glycogen with added free radioactive galactose results in the complete removal of this sugar during the early stages of purification. Galactose was identified by paper chromatography and by crystallization of the galactose methyl phenylhydrazone from acid hydrolyzates of the glycogen.

BIBLIOGRAPHY

1. Bernard, C. Compt. rend., 44, 578 (1857).

2. Haworth, W. N. and E. G. V. Percival. J. Chem. Soc., 2277 (1932).

3. Haworth, W. N., E. L. Hirst and F. A. Isherwood. J. Chem. Soc., 577 (1937).

4. Haworth, W. N., E. L. Hirst and F. Smith. J. Chem. Soc., 1914 (1939).

5. Manners, D. J. in Wolfrom, M. L. (editor) Advances in Carbohydrate Chem., Vol. 12, Academic Press, Inc., New York, 1957, p. 262.

6. Halsall, T. G., E. L. Hirst and J. K. N. Jones. J. Chem. Soc., 1399 (1947).

7. Liddle, A. M. and D. J. Manners. J. Chem. Soc., 3432 (1957).

8. Abdel-Ahker, M. and F. Smith. J. Am. Chem. Soc., 73, 994 (1951).

9. Cori, G. T. and J. Larner. J. Biol. Chem., 188, 17 (1951).

10. Larner, J. J. Biol. Chem., 202, 491 (1953).

11. Bridgman, W. B. J. Am. Chem. Soc., 64, 2349 (1942).

12. Greenwood, C. T. and D. J. Manners. Proc. Chem. Soc., 26 (1957).

13. Stetten, M. R., H. M. Katzen and D. Stetten, Jr. J. Biol. Chem., 222, 587 (1956).

14. Bryce, W. A. J., C. T. Greenwood, I. G. Jones and D. J. Manners. J. Chem. Soc., 711-15 (1958).

15. Stetten, D., Jr. and M. R. Stetten, in Springer, G. F. (editor) Polysaccharides in Biology. Transactions of the Third Conference 1957, Princeton, New Jersey, Vol. 3, Madison Printing Co., Inc., Madison, New Jersey, 1958, p. 9.

16. Stetten, M. R. and H. M. Katzen. J. Am. Chem. Soc., 83, 2912 (1961).

17. Green, A. A. and G. T. Cori. J. Biol. Chem., 151, 21 (1943).

18. Illingworth, B., D. H. Brown and C. F. Cori. Proc. Nat. Acad. Sci., 47, 469 (1961).

19. Brown, D. H., B. Illingworth and C. F. Cori. Proc. Nat. Acad. Sci., 47, 479 (1961).

20. Leloir, L. F. and C. F. Cardini. J. Am. Chem. Soc., 79, 6340 (1957).

21. Leloir, L. F., J. M. Olavarria, S. H. Goldemberg and H. Carminatti. Arch. Biochem. Biophys., 81, 508 (1959).

22. Wolfrom, M. L. and A. Thompson. J. Am. Chem. Soc., 79, 4214 (1957).

23. Thompson, A., K. Anno, M. L. Wolfrom and M. Inatome. J. Am. Chem. Soc., 76, 1309 (1954).

24. Sanger, F. Biochem. J., 39, 507 (1945).

25. Bell, D. J. J. Chem. Soc., 992 (1948).

26. Hirst, E. L., L. Hough and J. K. N. Jones. J. Chem. Soc., 928 (1949).

27. Boggs, L. A., L. S. Cuendet, I. Ehrenthal, R. Koch and F. Smith. Nature, 166, 520 (1950).

28. Abdel-Ahker, M. and F. Smith. Arch. Biochem. Biophys., 78, 451 (1958).

29. Wolfrom, M. L. and A. L. Thompson. J. Am. Chem. Soc., 77, 6403, (1955).

30. Bell, D. J. and D. J. Manners. J. Chem. Soc., 1891 (1954).

31. Peat, S., P. J. P. Roberts and W. J. Whelan. Biochem. J., 51, 17 (1952).

32. Bell, D. J. Biochem. J., 30, 1612 (1936).

33. Bell, D. J. Biochem. J., 30, 2144 (1936).

34. Halsall, T. G., E. L. Hirst and J. K. N. Jones. J. Chem. Soc., 1399 (1947).

35. Bacon, J. S. D., E. Baldwin and D. J. Bell. Biochem. J., 38, 198 (1944).

36. Whelan, W. J. and P. J. P. Roberts. Nature, 170, 748 (1952).

37. Illingworth, B. and G. T. Cori. J. Biol. Chem., 199, 653 (1952).

38. Illingworth, B., G. T. Cori and C. F. Cori. J. Biol. Chem., 218, 123 (1956).

39. Hauk, R., B. Illingworth, D.H. Brown and C. F. Cori. Biochem. Biophys. Acta, 33, 554 (1959).

40. Polglase, W. J., D. M. Brown and E. L. Smith. J. Biol. Chem., 199, 105 (1952).

41. Lewis, B. A. and F. Smith. J. Am. Chem. Soc., 79, 3929 (1957).

42. Whistler, R. L. and D. F. Durso. J. Am. Chem. Soc., 72, 677 (1950).

43. Cheronis, N. D. and J. B. Entriken. Semimicro Qualitative Analysis, Second Edition. Interscience Publishers, Inc., New York, 1958, p. 616.

44. Hassid, W. Z. and C. E. Ballou in Pigman, W. (editor) The Carbohydrates, Academic Press, Inc., New York, 1957, p. 498.

45. Hirst, E. L., J. K. N. Jones and E. A. Woods. J. Chem. Soc., 1048 (1947).

46. Cifonelli, J. A. and F. Smith. Anal. Chem., 27, 1639 (1955).

47. Jeanes, A., C. S. Wise and R. J. Dimler. Anal. Chem., 23, 415 (1951).

48. Partrige, S. M. Biochem. J., 42, 238 (1948).

49. Trevelyan, W. E., D. P. Proctor and J. S. Harrison. Nature, 166, 444 (1950).

50. King, E. J. Biochem. J., 26, 292 (1932).

51. Cardini, C. E. and L. F. Leloir, in Colowick, S. P. and N. O. Kaplan (editors), Methods in Enzymology, Vol. 3, Academic Press, Inc., New York, 1957, p. 843.

52. Park, J. T. and M. J. Johnson. J. Biol. Chem., 181, 149 (1949).

53. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith. Anal. Chem., 28, 350 (1956).

54. Umbreit, W. W., R. H. Burris and J. F. Stauffer. Manometric Techniques, Third Edition, Burgess Publishing Co., Minneapolis, Minnesota, 1957, p. 274.

55. Davidson, J. D. and P. Feigelson. Intl. J. Appl. Radiation and Isotopes, 2, 1 (1957).

56. Wood, W. A. Federation Proc., 19, 29 (1960).

57. Hassid, W. Z. and S. Abraham, in Colowick, S. P. and N. O. Kaplan (editors) Methods in Enzymology, Vol. 3, Academic Press, Inc., New York, 1957, p. 48.

58. Scott, H. M., B. C. Johnson and M. W. Moeller. Poultry Sci., 35, 924 (1956).

59. Somogyi, M. J. Biol. Chem., 104, 245 (1934).

60. Cifonelli, J. A., R. Montgomery and F. Smith. J. Am. Chem. Soc., 78, 2485 (1956).

61. Cifonelli, J. A., R. Montgomery and F. Smith. J. Am. Chem. Soc., 78, 2488 (1956).

62. Schachman, H. K. in Colowick, S. P. and N. O. Kaplan (editors) Methods in Enzymology, Vol. 4, Academic Press, Inc., New York, 1957, p. 32.

63. Bell, D. J., R. C. Gutfreund, R. Cecil and A. G. Ogston. Biochem. J., 42, 405 (1948).

64. Putman, E. W. in Colowick, S. P. and N. O. Kaplan (editors) Methods in Enzymology, Vol. 3, Academic Press, Inc., New York, 1957, p. 62.

65. Peat, S., W. J. Whelan, T. E. Edwards and O. Owen. J. Chem. Soc., 586 (1958).

66. Speck, J. C. in Wolfrom, M. L. (editor) Advances in Carbohydrate Chem., Vol. 13, Academic Press, Inc., New York, 1958, p. 63.

67. Dursch, H. R. and F. J. Reithel. J. Am. Chem. Soc., 74, 830 (1952).

68. Aspinall, G. O. in Wolfrom, M. L. (editor) Advances in Carbohydrate Chem., Vol. 14, Academic Press, Inc., New York, 1959, p. 429.

69. Aspinall, G. O., E. L. Hirst, E. G. V. Percival and I. R. Williamson. J. Chem. Soc., 3184 (1953).

70. Edelman, J. and J. S. D. Bacon. Biochem. J., 49, 446 (1951).

71. Hirst, E. L., D. I. McGilvary and E. G. V. Percival. J. Chem. Soc., 1297 (1950).

72. Palmer, A. Biochem. J., 48, 389 (1951).

73. Hehre, E. J. J. Biol. Chem., 222, 739 (1956).

74. Jeanes, A. J. Am. Chem. Soc., 76, 5041 (1954).

75. Peat, S., W. J. Whelan and H. G. Lawley. J. Chem. Soc., 724 (1958).

76. Goldstein, I. J., F. Smith and A. M. Unrau. Chem. and Ind. (London), 124 (1959).

77. Smith, F. and A. M. Unrau. Chem. and Ind. (London), 636 (1959).