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A PROTEIN-BOUND GLYCOGEN COMPONENT OF RAT LIVER*

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ABSTRACT

It has long been claimed, but frequently disputed, that part of the glycogen in rat liver is insoluble in 10% trichloroacetic acid, and a physiological significance was ascribed to the existence of the two pools of glycogen, desmo-glycogen, the insoluble form, and lyo-glycogen, the soluble component. Desmo-glycogen was thought to owe its acid insolubility to a covalent binding to protein. Recent claims that glycogen, similarly insoluble in acid, can be synthesized *in vitro* have renewed the interest in desmo-glycogen. We have obtained trichloroacetic acid-insoluble glycogen from rat liver and find that, despite subjecting the glycogen to proteolysis, peptide material remains in close association with the glycogen through a number of purification procedures and is freed from glycogen only by enzymic decomposition of the latter. The tenacity with which the glycogen and peptide material remain in association with each other is suggestive of the occurrence of protein-bound glycogen.

INTRODUCTION

The necessity for a carbohydrate primer in glycogen synthesis was discovered by Cori *et al.*[1]. Addition of a trace of glycogen was necessary for glycogen synthesis by muscle phosphorylase and branching enzyme, acting on α-D-glucopyranosyl phosphate, and the glycogen primer became an integral part of the glycogen product. Glycogen synthase, polymerizing UDP-D-glucose, also requires a carbohydrate primer[2]. Numerous investigations have been made as to the structurally most simple saccharide that will act as a primer. It seems that the malto-oligosaccharides—even maltose—will so function[3], and the synthesis of maltose has been decribed in a reaction between UDP-D-glucose and D-glucose catalyzed by glycogen synthase[4].

An alternative answer to the question of the origin of primer for glycogen and starch synthesis has been the subject of several recent reports (for a review, see ref. 5). The *in vitro* synthesis of glycogen and starch, both of them apparently attached to protein, has been claimed, in the case of glycogen, for crude enzyme systems from rat

*Dedicated to the memory of Professor J. K. N. Jones, F.R.S.

liver[6] and *Escherichia coli*[7], and from potato[8] in the case of starch. All three systems utilize UDP-D-glucose and/or ADP-D-glucose as the donor of D-glucose. The principal evidence for binding of the polysaccharide to protein is the precipitability of the synthetic polysaccharide by dilute trichloroacetic acid. Krisman and her colleagues[6] have postulated that the synthesis of protein-bound glycogen requires two proteins acting in cooperation with glycogen synthase and branching enzyme. The first participating protein acts as a primer. The second is an enzyme (glycogen initiator synthase) that adds D-glucosyl groups from the nucleoside 5′-(D-glucopyranosyl diphosphate) substrate to the protein primer to form short, protein-bound, malto-oligosaccharide chains that are then extended by glycogen synthase.

These reports recall the extensively investigated claim that there are two forms of mammalian glycogen: desmo-glycogen, which is protein-bound (trichloroacetic acid-precipitable) and lyo-glycogen, which is not protein-bound (for a review, see ref. 9). Little has been reported on the subject during the past 15 years, probably out of a persuasion that the two forms of glycogen were artefacts[10]. In view of the reports of the *in vitro* synthesis of protein-bound glycogen, we decided to re-investigate the question of the presence of a protein-bound glycogen component in rat liver.

MATERIALS AND METHODS

Preparation of a "glycogen–peptide" from rat liver. — Three male rats (about 325 g each) were starved overnight and then fed Purina rat chow 1 h before being decapitated. The livers (40 g total) were homogenized in 10 vol. of ice-cold 10% trichloroacetic acid and centrifuged. The sediment was suspended for 1 h each in the following solvents (100 ml) with occasional shaking, centrifuging between each solvent change: methanol, diethyl ether, 2:1 (v/v) chloroform–methanol (twice), 10:5:0.3 (v/v) chloroform–methanol–water, and methanol. The residue, dried in a desiccator under vacuum, weighed 8 g. The protein component of the residue was then subjected to proteolysis in a denaturing solvent. The residue (2.8 g) in 100 ml of solution (pH 8.0) containing 6M guanidinium hydrochloride, 0.1M Tris, 0.1M potassium chloride, 0.01M calcium chloride, and Chaotropase (20 mg, Calbiochem)[11], was incubated for 48 h at 25°. The solution was dialyzed for a few h each against three changes of water (6 l each). A sediment formed that was not removed. The suspension was freeze-dried and subjected to further proteolysis. It was suspended in 25 ml of 0.1M Tris buffer, pH 8.0, containing 5mM calcium chloride and 200 mg of Pronase (Calbiochem, B grade), being incubated for 48 h at 37°. The digest was centrifuged and the clear solution dialyzed for a few h each against 6 l of 0.1M sodium chloride followed by three changes of water (6 l). The solution was passed through a column of DEAE-cellulose Whatman DE 52 (1.6 × 10 cm) previously equilibrated with 0.2M potassium phosphate buffer, pH 7.0, and was washed through with three bed-volumes of the same buffer. This displaced the "glycogen–peptide" and served to retain nucleic acids. The combined eluate was dialyzed against three changes of water (6 l each), freeze-dried, dissolved in 2 ml of water, applied to a column of Bio-

TABLE I

AMINO ACID COMPOSITION OF THE RAT-LIVER "GLYCOGEN–PEPTIDE"

Amino acid	*Amount*[a]	*Amino acid*	*Amount*[a]
Alanine	3.9	Lysine	1.3
Aspartic acid	9.0	Phenylalanine	1.0
Glycine	5.6	Proline	4.6
Glutamic acid	4.7	Serine	7.1
Histidine	2.0	Threonine	6.9
Isoleucine	2.7	Tyrosine	1.1
Leucine	3.6		

[a] The results are expressed relative to phenylalanine (1.0). The sample that was analyzed contained 6.0 μmol of D-glucose equivalents by phenol–sulfuric acid determination and 25.3 nmol of amino acid residues, of which 3.36 were serine.

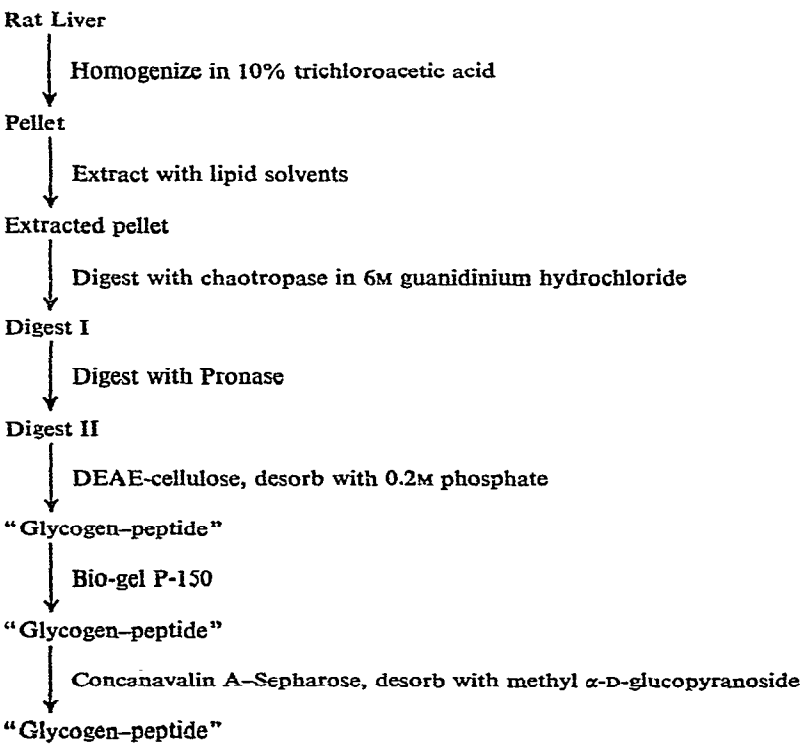

Fig. 1. Purification of a "glycogen–peptide" from rat liver. At the last stage of purification, following desorption of the "glycogen–peptide" from concanavalin A–Sepharose, the product was labelled with tritiated 1-fluoro-2,4-dinitrobenzene. Full experimental details are given in Materials and Methods.

Gel P-150 (Bio-Rad Laboratories, 1.6 × 60 cm) equilibrated in 0.1M ammonium hydrogencarbonate, and eluted with the same solution. The void-volume fractions were pooled and freeze-dried. The powder was dissolved in 1 ml of M sodium chloride containing 20mM potassium phosphate (pH 7.0), and applied to a column of concanavalin A–Sepharose (Pharmacia, 1.6 × 15 cm). The column was washed with 100ml of the same buffer and the "glycogen–peptide" eluted with 60 ml of the same buffer made 0.1M in methyl α-D-glucopyranoside. The methyl α-D-glucopyranoside eluate was dialyzed against three changes of water (6 l each) and freeze-dried. The glycogen content of the product, determined enzymically[12], was 16.5 mg. It was analyzed for its content of amino acids (Table I). The glycogen-purification scheme is shown in Fig. 1.

Reaction of the "glycogen–peptide" with ^{3}H-labelled 1-fluoro-2,4-dinitrobenzene. — All procedures involving derivative formation with 1-fluoro-2,4-dinitro-[3,5-^{3}H]-benzene (New England Nuclear 23.6 Ci/mmol) were conducted in the dark as described by Sanger[13]. The "glycogen–peptide" was dissolved in 2 ml of 5% sodium hydrogencarbonate; a solution of 1-fluoro-2,4-dinitro[^{3}H]benzene (0.25 mCi, 10 nmol) in benzene (1 ml) and ethanol (3 ml) was then added. The mixture was shaken for 24 h at room temperature. Ethanol (15 ml) was added and the mixture centrifuged, washed twice with ethanol (15 ml each) and suspended in diethyl ether (14 ml). Water (2 ml) was added to dissolve the residue, the ether layer was removed, and the sample was precipitated with ethanol (14 ml). The precipitate, collected in a centrifuge tube, was dissolved in 2 ml of water, extracted with ether (20 ml), precipitated with ethanol, and the cycle repeated twice more. Finally the precipitate, in water (1 ml), was applied to a column (1.6 × 60 cm) of Bio-Gel P-150 and eluted with 0.1M ammonium hydrogencarbonate buffer. Fractions were collected and their ^{3}H contents determined in a Packard Model 3380 liquid scintillation-counter (Fig. 2). Those void-volume fractions containing ^{3}H (nos. 10–20) were pooled and freeze-dried and dissolved in 2 ml of water. The solution was kept frozen until required.

Molecular sieving of the "glycogen–peptide" before and after glycogenolysis. — A portion of the solution of ^{3}H-labelled "glycogen–peptide" (0.1 ml) was digested with *Aspergillus niger* glucoamylase deliberately adulterated with a trace of alpha amylase (*Bacillus subtilis*) so that complete glycogenolysis would be assured[14]. The sample, and an untreated control, were passed through a column (1.6 × 60 cm) of Bio-Gel P-150 and eluted with 0.1M sodium hydrogencarbonate, pH 8.0. The tritium content of the fractions (1.6 ml each) was determined (Fig. 3).

Equilibrium density-gradient centrifugation[15,16] of the "glycogen–peptide". — The ^{3}H-labelled "glycogen–peptide" (0.5 ml) was freeze-dried and dissolved in 4 ml of 4M guanidinium chloride (Schwarz–Mann, Ultrapure) containing 0.597 g of cesium sulfate (Kawecki Berylco Industries, Rare Metals Division) per ml, and centrifuged for 48 h at 4° and 125,000 *g* in a Beckman–Spinco L2-65B ultracentrifuge in an SW 56 Ti rotor. After centrifugation, the tube was punctured at the bottom; 4-drop fractions were collected, and their content of ^{3}H determined. The same treatment was given to a sample of "glycogen–peptide" pretreated with glucoamylase (see

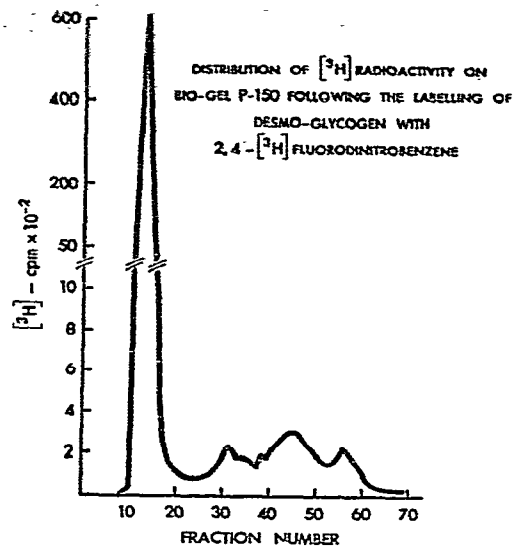

Fig. 2. The distribution of ^3H in fractions (1.6 ml each) collected from Bio-Gel P-150 when "glycogen–peptide" treated with tritiated 1-fluoro-2,4-dinitrobenzene was passed through a gel column (1.6 × 60 cm). Fractions 10–20 were used in the experiments described in Figs. 3–5 and Tables I and II. For details, see Materials and Methods.

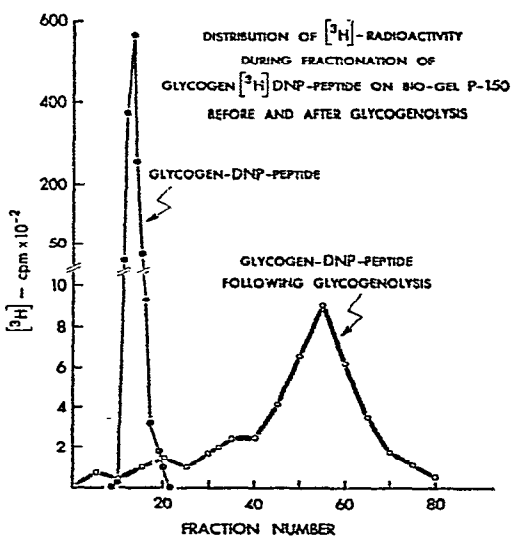

Fig. 3. The distribution of ^3H in fractions (1.6 ml each) collected from Bio-Gel P-150 when "glycogen–peptide" (Fig. 2) was passed through a column (1.6 × 60 cm) before (solid circles) or after (open circles) treatment with glucoamylase/alpha amylase. For details, see Materials and Methods. DNP = dinitrophenylated.

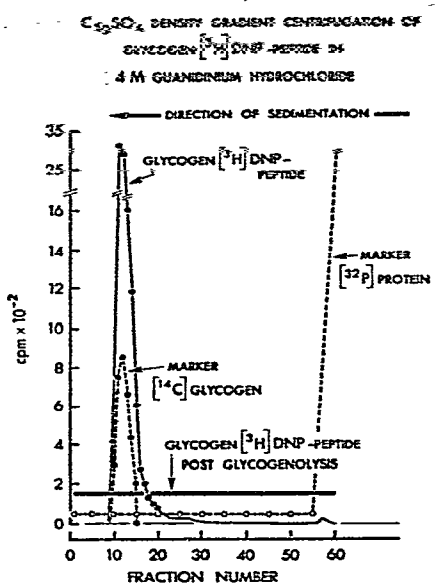

Fig. 4. Cesium sulfate density-gradient centrifugation of tritiated "glycogen–peptide" (Fig. 2) before and after treatment with glucoamylase/alpha amylase (Fig. 3). ^{14}C-Labelled glycogen and ^{32}P-labelled protamine were included as marker substances. Fractions were collected from the bottom of the centrifuge tube after 48 h of centrifugation. For details, see Materials and Methods. DNP = Dinitrophenylated.

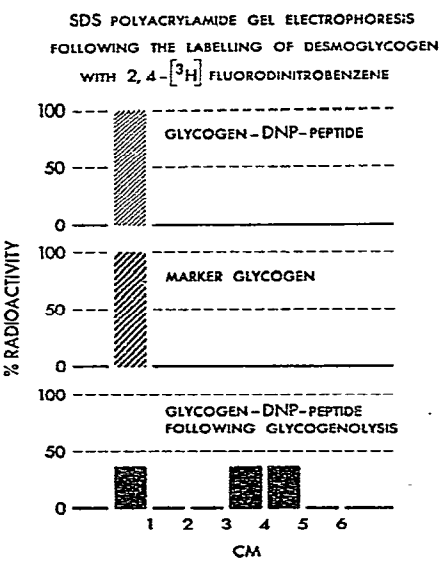

Fig. 5. Poly(acrylamide)-gel electrophoresis of tritiated "glycogen–peptide" (Fig. 2) before and after treatment with glucoamylase/alpha amylase (Fig. 3). ^{14}C-Labelled glycogen was also subjected to electrophoresis for comparison. For details, see Materials and Methods. DNP = Dinitrophenylated.

above), to a sample of rabbit-liver glycogen labelled with ^{14}C from UDP-D-[^{14}C]-glucose by liver glycogen synthase[17], and to ^{32}P-labelled protamine. The results are shown in Fig. 4.

Gel electrophoresis of the "glycogen–peptide". — Polyacrylamide gel-electrophoresis in sodium dodecyl sulfate was performed as described by Shapiro *et al.*[18] using 5% acrylamide. The sample (0.1 ml) and tracking dye were mixed with 0.1 ml of 10% acrylamide and polymerized onto the surface of the gels. A current of 4 mA was passed through the gel until the tracking dye reached the bottom. The gel was then sliced into 1-cm segments and the ^{3}H or ^{14}C in each segment determined. The samples that were so submitted to electrophoresis were the same as those subjected to density-gradient centrifugation (see Fig. 5).

Amino acid analysis of the "glycogen-peptide". — The "glycogen–peptide" (0.8 mg) was hydrolyzed for 24 h in a solution (1 ml) of 6M hydrochloric acid and 1% phenol at 105° in a sealed, evacuated tube. The resulting mixture of amino acids was resolved and their amounts were determined in a Durrum D500, automatic amino-acid analyzer (see Table I).

Chain extension of the "glycogen–peptide". — The "glycogen–peptide" (0.5 ml) was freeze-dried and dissolved in a solution (1 ml) containing 150 μmol of α-D-[^{14}C]-glucopyranosyl phosphate (4×10^{6} counts/min; New England Nuclear), 40mM imidazole, pH 6.5, and 150 units[19] of rabbit-muscle phosphorylase *a*. A precipitate formed during incubation at room temperature for 1 h. This was collected by centrifugation, washed three times with water (12 ml each time), and dissolved in dimethyl sulfoxide (2 ml). A portion (20 μl) of the solution was used to determine the content of ^{3}H and ^{14}C by using the dual-label counting channels of a Packard model 3380 liquid scintillation-counter. The polysaccharide was precipitated with 4 vol. of ethanol, centrifuged, redissolved in dimethyl sulfoxide, and reprecipitated 8 times, determining ^{3}H and ^{14}C each time the precipitate was redissolved. The results are shown in Table II.

TABLE II

RECOVERY[a] OF ^{3}H AND ^{14}C DURING REPRECIPITATION OF "GLYCOGEN–PEPTIDE" LABELLED WITH ^{3}H IN THE PEPTIDE AND ^{14}C IN THE CARBOHYDRATE

Precipitation no.	^{3}H (10^{6} counts/min)	^{14}C (10^{6} counts/min)	^{3}H:^{14}C
1	3.66	0.80	4.58
2	4.10	1.06	3.87
3	2.76	1.02	2.71
4	3.36	1.08	3.11
5	3.09	1.08	2.86
6	3.10	0.99	3.13
7	2.81	0.90	3.12
8	2.90	0.95	3.05
9	2.82	0.91	3.10

[a]The radioactivity was measured after each precipitate had been redissolved in dimethyl sulfoxide.

Reaction of "protein-free" glycogen with ^{3}H-labelled 1-fluoro-2,4-dinitrobenzene. — Shellfish glycogen (Schwarz–Mann) was treated to remove protein both by physical and chemical manipulation. A 10% solution of the glycogen was mixed with an equal volume of 10% trichloroacetic acid. The sediment was removed by centrifugation and the glycogen precipitated with 4 vol. of ethanol. The glycogen was redissolved and reprecipitated twice more with ethanol, and then dried by washing successively with ethanol and diethyl ether, and removing the solvent in a vacuum. The glycogen (2 g) was dissolved in 5M potassium hydroxide (20 ml) and heated for 2 h in a boiling-water bath. It was twice precipitated with ethanol as before, dissolved in water (100 ml), dialyzed overnight against water (4 l), and recovered as a dried powder as already described.

A sample of the alkali-treated glycogen (16.5 mg) was treated with tritiated 1-fluoro-2,4-dinitrobenzene as described for the "glycogen–peptide". At the end of the reaction, the glycogen was recovered by addition of 2 ml of 0.8% carrier glycogen followed by 15 ml of ethanol. The precipitate was redissolved in water (2 ml), reprecipitated four times, and dissolved in 4 ml of water. At this point, the total ^{3}H content was 214,100 counts/min. After dialysis overnight against 1 l of water, the ^{3}H content was 109,000 counts/min.

RESULTS AND DISCUSSION

In attempting to learn whether rat liver contains a protein-bound glycogen component, the strategy we employed was to take the fraction of rat liver insoluble in 10% trichloroacetic acid, namely the classical source of "desmo-glycogen", and to digest this fraction with proteases so that we were likely to obtain a water-soluble glycogen, the molecules of which were possibly terminated by peptides. The glycogen was then fractionated by the several procedures shown in Fig. 1. Nucleic acids were removed on DEAE-cellulose, and material included in Bio-Gel P-150 (exclusion limit mol. wt. 150,000) was eliminated. The glycogen was then adsorbed on concanavalin A covalently bound to Sepharose, and selectively desorbed with methyl α-D-glucopyranoside. The glycogen content of the eluted product was 16.5 mg, as determined by an enzymic procedure specific for glycogen. It amounted to 0.12% of the original wet weight of liver taken. There was no significant content of other carbohydrate, because an assay of total carbohydrate made by the non-specific phenol–sulfuric acid method[20] gave the same value as in the glycogen-specific assay.

The glycogen preparation was then analyzed for amino acids. These were present to the extent of 1 amino acid residue per 237 D-glucose residues (Table I). The major amino acids were serine and threonine, both implicated in mammalian glycopeptide bonds, and aspartate, also implicated as asparagine. The number of D-glucose residues relative to each mole of aspartate was 1410. The use of these data to calculate a molecular weight of the glycogen could give meaningless values as (*a*) the peptide material, being a digestion product, is unlikely to have a unique constitution, and (*b*) the method of preparation of the material would not exclude contamination by peptide-free glycogen.

Attempts were made to separate the glycogen from the peptide material that had accompanied it to this point. These were by molecular sieving, density-gradient centrifugation, and gel electrophoresis. It was necessary to introduce a marker so that the peptide could be detected, and this was achieved with ^{3}H-labelled 1-fluoro-2,4-dinitrobenzene, which reacts with amino, phenolic, imidazole or sulfhydryl groups. When the derivative was passed through Bio-Gel P-150, virtually all of the label was found in the excluded fraction (Fig. 2), together with the glycogen. When the glycogen was first degraded with glucoamylase, the label penetrated the gel (Fig. 3). Similarly, the ^{3}H label cosedimented with marker glycogen in a gradient of cesium chloride (Fig. 4). When the glycogen was first degraded with glucoamylase, the label was then distributed uniformly throughout the gradient (Fig. 4). The ^{3}H-labelled "glycogen–peptide", like marker glycogen, did not penetrate a polyacrylamide gel under denaturing conditions, but two-thirds of the label did so after glycogenolysis (Fig. 5).

In a control experiment, glycogen that had been treated with trichloroacetic acid and boiling alkali, to remove any proteinaceous material, was then treated with tritium-labelled 1-fluoro-2,4-dinitrobenzene, as for the "glycogen–peptide". The amount of ^{3}H incorporated into the protein-free glycogen was only 1.2% of that incorporated into an equal weight of the "glycogen–peptide".

These results are uniformly consistent with our having obtained a glycogen fraction covalently bound to peptide material. The peptide material itself, when freed from glycogen, is seen to have a relatively low molecular weight (Figs. 3–5). But, when the glycogen is left intact, the peptide material accompanies it through fractionation on concanavalin A (Fig. 1) on Bio-Gel P-150 (Figs. 2, 3), in a density gradient (Fig. 4), or during electrophoresis under denaturing conditions (Fig. 5).

Finally, an attempt was made to separate the glycogen from the peptide material by taking advantage of the fact that, when glycogen chains are elongated with muscle phosphorylase and α-D-glucopyranosyl phosphate, and branching enzyme is absent, a product is formed that is extremely insoluble in water. It may be dissolved in dimethyl sulfoxide. When the experiment was performed with ^{14}C-labelled α-D-glucopyranosyl phosphate to detect the elongated chains, 93% of the ^{3}H label was recovered in the redissolved precipitate. The polysaccharide was precipitated with ethanol from dimethyl sulfoxide a further eight times. The ^{3}H and ^{14}C contents of the redissolved material were measured each time. From the third to the ninth precipitation, the recovery of each isotope and the ratio of ^{3}H to ^{14}C remained essentially constant (Table II), indicating the firm association between the ^{14}C-labelled carbohydrate and the ^{3}H-labelled peptide.

We consider that the foregoing experiments offer evidence for the existence in rat liver of desmo-glycogen, and give physiological significance to claims of other workers[6–8] to have synthesized protein-bound glycogen *in vitro*. We propose next to determine the chemical nature of the glycogen–protein bond, with due attention to the possibility that sugars other than D-glucose, for instance D-xylose and/or 2-amino-2-deoxy-D-glucose, may intervene between glycogen and the protein.

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