Carbohydrate Research, 149 (1986) 35–45
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

A (1→4)-α-D-GLUCAN–PROTEIN INVOLVED IN LIVER GLYCOGEN BIOSYNTHESIS*,†

CLARA R. KRISMAN‡, ROBERTO A. GEREMIA§,
Instituto de Investigaciones Bioquímicas "Fundacion Compamar", Facultad de Ciencias Exactas y Naturales and CONICET, Antonio Machado 151 (1405), Buenos Aires (Argentina)

AND WILLIAM J. WHELAN
Department of Biochemistry, University of Miami School of Medicine, Miami, Florida 33101 (U.S.A.)
(Received February 1st, 1985; accepted for publication, April 3rd, 1985)

ABSTRACT

A macromolecular (1→4)-α-D-[^{14}C]glucan–protein complex was synthesized with a rat liver preparation and uridine diphosphate D-[^{14}C]glucose. The size of the complex is contributed by both the protein and the (1→4)-α-D-glucosyl-oligomer components. Iodoacetamide treatment did not change the migration properties on Bio-Gel A-50m. Therefore, disulfide bonds linking glucan–protein subunits seem not to be involved. The [^{14}C]glucan–protein, precipitated by diluted trichloroacetic acid, was digested by α-amylase, phosphorylase *a*, and proteases. The extent of proteolysis was greater for a complex having fewer D-glucose units incorporated. After proteolytic digestion of that complex, the labeled fragments behaved on electrophoresis, and ion-exchange and gel chromatography as [^{14}C]glucosylated peptides. These findings support previous conclusions that the primer for liver glycogen synthesis is a protein on which glycogen is built up by covalent attachment.

INTRODUCTION

The long-debated question whether mammalian glycogen contains a covalently bound protein component[1] was reopened by one of us[2–4] with the demonstration that a pellet sedimented from a rat-liver extract is able to incorporate D-glucose from UDP-glucose into a product that is precipitated by dilute trichloroacetic acid and appears to contain protein. The glucose units so incorporated are linked

*Dedicated to Dr. Luis F. Leloir on the occasion of his 80th birthday.
†Part of this work was presented, as a poster, at the IVth Congress of PAABS (The Pan-American Association of Biochemical Societies), Buenos Aires, November 4–8, 1984. The work was supported, in part, by a grant from the U.S. National Institutes of Health (AM-12532).
‡Career Investigator of the Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina (CONICET).
§Fellow of the Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina (CONICET).

0008-6215/86/$ 03.50

through α-(1→4) bonds, as shown by their removal, as maltose, by α-amylase[2,4]. The enzyme preparation is free from branching enzyme[4], so that it appears that the D-glucan chains are linear. Hence, we refer to them as glucans rather than glycogen.

Similar observations have been made with other glycogen- and starch-synthesizing systems. Thus, enzyme preparations from heart tissue[5], *Escherichia coli*[6], *Neurospora crassa*[7,8], and potatoes[9] are described as giving rise to trichloroacetic acid-insoluble, ^{14}C-labeled products when incubated with the appropriate radiolabeled nucleoside diphosphate sugar, UDP-glucose or ADP-glucose. That such products synthesized *in vitro* have a physiological relevance is indicated by reports of the occurrence, in heart tissue[10] and rat liver[11], of trichloroacetic acid-insoluble glycogen containing protein that is seemingly covalently bound to the glycogen. A (1→4)-α-D-glucan, also protein-bound, has been reported in rabbit muscle[12] and in bovine retinal membranes[13].

To explain these observations, Krisman and Barengo[4] postulated that the protein constitutes the initiation point for glycogen synthesis, acting as a primer on which a putative enzyme, "glycogen-initiator synthase", constructs maltosaccharide chains that in turn prime the synthesis of glycogen by glycogen synthase (EC 2.4.1.11) and branching enzyme (EC 2.4.1.12). The foregoing reports of the occurrence of protein-bound glucose in heart, liver, muscle, and retina would suggest that the protein persists in the final product rather than acting as a transient biosynthetic intermediate. Further studies on the [^{14}C]glucan–protein synthesized by rat-liver enzyme *in vitro* in order to test for covalent association between the glycogen and protein are reported herein. These studies confirm that the *in vitro*-synthesized glucan–protein is of high-molecular weight[14]. After extensive proteolytic digestion of the complex, [^{14}C]glucosylated peptides have been detected, indicating that a (1→4)-α-D-[^{14}C]glucan is covalently linked to a protein in such a complex.

EXPERIMENTAL

Chemicals. — UDP-D-[U-^{14}C]glucose (11.4 MBq/mol) was prepared as described by Thomas *et al.*[15]. Unlabeled UDP-glucose, Blue Dextran, and Tris were from the Sigma Chemical Co. Bio-Gel A-50m (100–200 mesh) and glycine were purchased from Bio-Rad Laboratories. Sucrose was obtained from Schwarz–Mann and Sephadex G-75 from Pharmacia Fine Chemicals.

Enzymes. — The rat-liver enzyme preparation was obtained and assayed as previously described[2–4]. Bacterial α-amylase (EC 3.2.1.1), insoluble α-amylase, and proteases types VIII and VI were purchased from Sigma.

Chaotropase was kindly provided by Dr. W. M. Awad (University of Miami)[16]. Muscle phosphorylase *a* (EC 2.4.1.1) (2000 U/ML; specific activity 80 U/mg, maintained in 50% glycerol–50mM imidazole–5mM theophylline–mM D,L-dithiothreitol, pH 7.2) was a gift of Dr. Derek Killilea (University of Miami).

The proteases were free from detectable amylolytic activity when tested with

protein-free [^{14}C]glycogen as substrate. The glycogen was incubated under optimal protease conditions for 48 h. Portions of the digests were removed at intervals and the glycogen precipitated with ethanol (3 vol.) in the presence of ammonium acetate. The ^{14}C-radioactivity values in the precipitate and supernatant were measured. No loss of ^{14}C-radioactivity from the precipitate to the supernatant was noted.

Analytical procedures. — Protein was determined according to Bradford[17] and radioactivity was measured in aqueous samples dissolved in Bray's solution[18].

D-[^{14}C]*Glucan–protein synthesis.* — A mixture containing 10mM glycine–NaOH buffer (pH 8.7), 100mM Na EDTA (pH 8.0), 5mM dithiothreitol, mM UDP-[^{14}C]glucose (specific activity 5.1×10^6 c.p.m./μmol), and liver enzyme (~2 mg of protein) was incubated at 37° for 45 min, in a total volume of 0.1 mL. Immediately after incubation, the mixture was applied to a Sephadex G-75 column (1 × 26 cm) equilibrated with 50mM Tris · HCl buffer (pH 8.2) and fractions (0.5-mL) were collected. The radioactive effluents excluded from the column (fractions 4–6, Fig. 1) were pooled and precipitated with cold trichloroacetic acid added to give a 10% concentration. After centrifugation, the precipitate was washed several times with 10% trichloroacetic acid and ether, and dried *in vacuo*. The ^{14}C-containing material recovered showed a radioactivity of ~300 000 c.p.m. This excluded fraction, obtained as described, is the glucan–protein used in the following experiments.

Proteolytic digestions. — The [^{14}C]glucan–protein (310 000 c.p.m.) was digested with chaotropase (2 mg) in 100mM Tris · HCl buffer (pH 8.1) containing[19] 25mM $CaCl_2$ and 6M guanidine · HCl or with protease type VI or type VIII (2 mg) in 100mM Tris · HCl buffer (pH 8.1) containing[19] 5mM $CaCl_2$. The total volume was 0.2 mL and the incubations were performed at 37° under toluene vapors for 9 days. Every 24 h, the mixtures were supplemented with the corresponding solid protease preparation (1–2 mg).

Glucan digestions. — The [^{14}C]glucan–protein (310 000 c.p.m.) was incubated with phosphorylase *a* (1 mg), mM 5′-AMP, mM dithiothreitol, and 100mM phosphate buffer (pH 7.8) at 37°, in a total volume of 0.8 mL. Alternatively, the [^{14}C]glucan–protein was incubated with soluble (0.195 U) or insoluble (0.33 mg) α-amylase in 10mM Tris · HCl buffer (pH 7.2) at 37°, in a total volume of 0.8 mL. The times of incubation are shown in Fig. 6.

Bio-Gel A-50m chromatography. — A Bio-Gel A-50m (100–200 mesh) column (1 × 32 cm) was equilibrated and eluted with 100mM $(NH_4)_2CO_3$ (pH 9.0). The void volume was determined with either a selected, very high-molecular-weight liver glycogen population[20] or a suspension of *Rhizobium meliloti* containing 6×10^8 bacteria per mL. The presence of glycogen in the effluent was determined with the $CaCl_2$–I_2 reagent[21] and that of bacteria was monitored by the absorbance at 660 nm.

Iodoacetamide treatment of [^{14}C]*glucan–protein.* — Essentially, the procedure of Geddes *et al.*[22] was followed. The vacuum-dried [^{14}C]glucan–protein sample (310 000 c.p.m.) was resuspended in 100mM Tris · HCl buffer (0.2 mL, pH 8.5)

containing 100mM dithiothreitol and 8M urea. After incubation for 4 h at 37°, iodoacetamide (14.8 mg) was added and the mixture was kept for 15 min at room temperature, the pH being maintained between 8.2–8.5 by addition of M NH_4OH. The reaction was stopped with concentrated 2-mercaptoethanol (0.5 mL), and the mixture dialyzed overnight against 10mM $(NH_4)_2CO_3$ (600 mL, pH 9.0). The retentate was evaporated to dryness and the residue dissolved in 10mM $(NH_4)_2CO_3$ (0.2 mL, pH 9.0). This solution was chromatographed on Bio-Gel A 50m as described earlier. An untreated [^{14}C]glucan–protein sample directly applied to a Bio-Gel A 50m column was used as control.

Ion-exchange chromatography. — The [^{14}C]glucose-labeled material was digested with type VIII protease as described earlier, lyophilized, and redissolved in a small volume of distilled water. The pH was adjusted to 2 with 0.01M HCl. The solution (1 mL) was applied to a Dowex 50W-X8 column (0.7 × 60 cm) equilibrated with 0.2M pyridine acetate buffer (pH 3.0). The column was washed with the same buffer and then connected to two consecutive linear gradients (200 mL each); the first one was from 0.2M (pH 3) to 0.5M (pH 5) pyridine acetate, and the second one was at pH 5 from 0.5M to 5M pyridine acetate. All these steps were carried out at 4° due to the acid lability of the compound at higher temperatures. Fractions (1 mL) were collected and aliquots (0.1 mL) were mixed with Bray's scintillation solution (3.5 mL each) and the radioactivity was counted with a Beckman 8100 liquid-scintillation counter.

The radioactive material that was not bound to the resin was lyophilized and digested overnight with insoluble α-amylase at 37° under toluene vapors. The insoluble α-amylase was removed by centrifugation and the pH of the supernatant adjusted to 2 with 0.01M HCl. The acidified supernatant was again submitted to ion-exchange chromatography as just described.

Paper electrophoresis. — Paper electrophoresis was performed according to Markham and Smith[23] with minor modifications. Whatman No. 1 paper strips (60 × 2.5 cm) soaked in the running buffer were used for each sample. The mixture was applied in the middle of the strip. The reducing substances were developed[24] with alkaline $AgNO_3$. Electroendosmosis was determined by following the migration of D-glucose and the radioactivity as follows. After electrophoresis the papers were dried at room temperature, and then cut into strips (1 × 2.5 cm) and placed into toluene-scintillation solution and counted. As formic acid is a volatile buffer, the strips containing radioactivity were then washed with methanol, acetone, and ether and dried. The radioactive material was eluted with distilled water and then submitted to the second electrophoresis in buffer B, as described in the legend to Fig. 5.

RESULTS AND DISCUSSION

Rat-liver extracts were incubated with UDP-[^{14}C]glucose and the mixture was subjected to gel filtration. The elution profile of the [^{14}C]glucan–protein from a

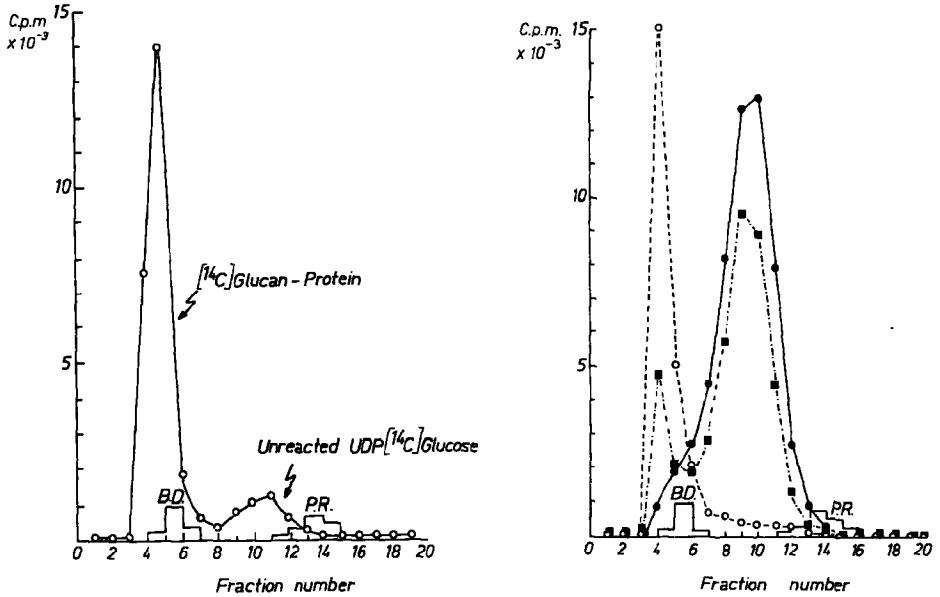

Fig. 1. Sephadex G-75 chromatography of a standard incubation mixture in which [^{14}C]glucan–protein had been synthesized. Conditions were as described in the Experimental section. Radioactivity was determined in a 50-μL aliquot of each 500-μL fraction. B.D., Blue Dextran; P.R., Phenol Red.

Fig. 2. Gel filtration on Sephadex G-75 of the proteolytic digestion products of [^{14}C]glucan–protein (310 000 c.p.m., Fig. 1) with proteolytic enzymes as described in the Experimental sections B.D., Blue Dextran; P.R., Phenol Red; (—○—○—) chaotropase, (—●—●—) protease type VIII, and (—■—■—) protease type VI.

Sephadex G-75 column is depicted in Fig. 1. As previously shown[4], the turbid material containing the ^{14}C-labeled product was eluted in the void volume. The distribution of radioactivity between included and excluded fractions indicated that the conversion of UDP-[^{14}C]glucose into the compound was rather complete.

In order to gain further insight into the structure of the glucan–protein, it was incubated with proteolytic enzymes. The products from protease types VI and VIII treatment were included in Sephadex G-75 (Fig. 2). Under similar conditions, the products obtained after chaotropase treatment were not so included (Fig. 2). The latter products were next submitted to gel-permeation chromatography on Bio-Gel A-50m. As shown in Fig. 3, the [^{14}C]glucan–protein had been digested by chaotropase, but the products were evidently larger than those obtained with protease types VI and VIII.

The products generated after exhaustive incubation of the [^{14}C]glucan–protein complex with protease type VIII were chromatographed on Dowex 50W-X8 as described in the Experimental section. Two peaks containing radioactive material were obtained, the first one being eluted from the column at the very beginning and the second one with 0.34M pyridine acetate (data not shown). Incubation of the first peak with insoluble α-amylase gave again radioactive-labeled

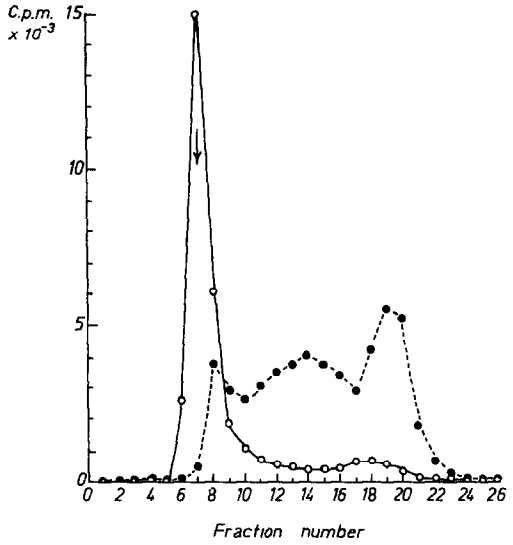

Fig. 3. Bio-Gel A-50m chromatography of [^{14}C]glucan–protein before (—○—○—) and after digestion with chaotropase (--●--●--). The [^{14}C]glucan–protein obtained as the excluded fraction, in the experiment described in Fig. 1, was dissolved in 100mM ammonium carbonate (200 μL, pH 9.0) and applied to a Bio-Gel A-50m column (1 × 32 cm). The digestion products obtained after incubation with chaotropase were filtered under similar conditions. The arrow indicates the void volume. Radioactivity was determined by taking a 100-μL aliquot of each 500-μL fraction before or a 200-μL aliquot after chaotropase treatment, and counting directly after addition to Bray's solution (3.5 mL). Other conditions are described in the Experimental section.

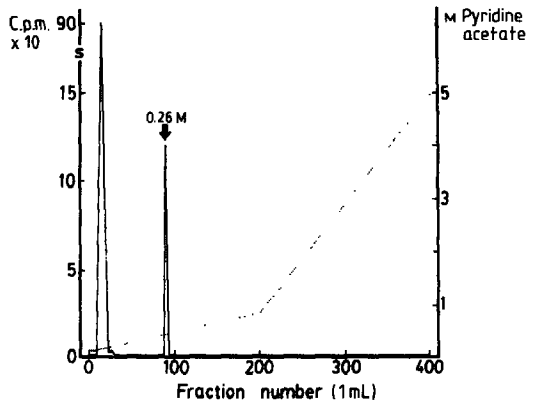

Fig. 4. Ion-exchange chromatography, on a Dowex 50W-X8 column as described in the Experimental section, of the [^{14}C]glucan–protein complex submitted to proteolysis. The first peak containing radioactive material, eluted with the washing buffer and then incubated with insoluble α-amylase, was loaded onto the column and chromatographed as described in the Experimental section: (——) Counts/min × 10; (·····) molarity of pyridine acetate.

compounds, part of which were retained in the Dowex 50W-X8 column and eluted at 0.26M pyridine acetate (Fig. 4). These results clearly showed that it is necessary to shorten the glucan branches in order to bind the glucan–peptide to the column. When the ^{14}C-labeled compounds, eluted with 0.26M pyridine acetate, were submitted to paper electrophoresis, they proved to be amphoteric (Fig. 5). They migrated towards the positive pole in an alkaline buffer (pH 10) and to the negative pole in acid medium (pH 2). Thus, they had the properties of amino acid-containing compounds.

We have observed, in unreported experiments, that when fewer glucose residues are incorporated into the glucan–protein, the extent of proteolysis is greater. Evidently, the glucan moiety offers steric hindrance to the protease. Since, after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (experiments not reported here), the [^{14}C]glucan–protein complex remained in the upper portion of the gel and, as shown in Fig. 3, the glucose–protein appeared to have a high-molecular weight, experiments were carried out to determine the contribution of protein and polysaccharide to the size of the glucan–protein complex. The approach used was to treat the compound with a disulfide-bond-breaking reagent in order to dissociate the protein component, or in turn to degrade the polysaccharide part of the glucan–protein molecule with phosphorylase *a* or α-amylase.

Glucan–protein treated with iodoacetamide migrated on Bio-Gel A 50m as

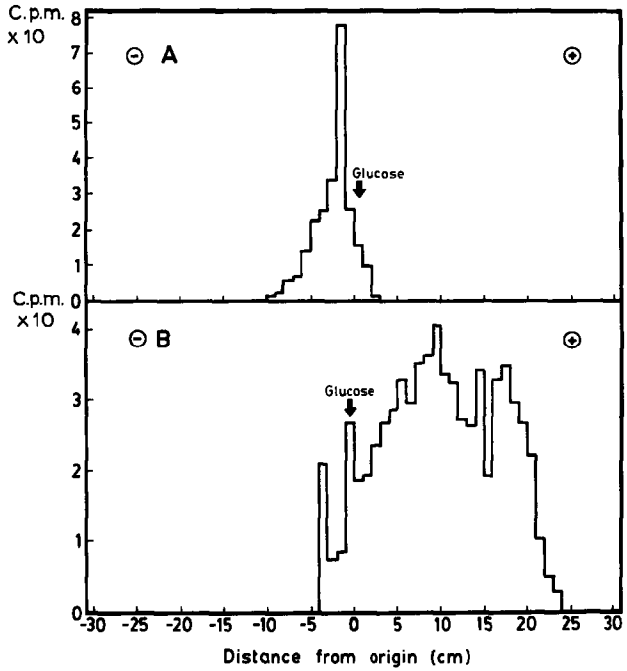

Fig. 5. Paper electrophoresis of the peak eluted from the Dowex 50W-X8 column. An aliquot of the peak eluted with 0.26M pyridine acetate (see Fig. 4) was applied. Electrophoresis (2 h at 17 V/cm) was carried out in: (A) 5% formic acid (pH 2) and (B) 0.2M sodium hydrogencarbonate (pH 10).

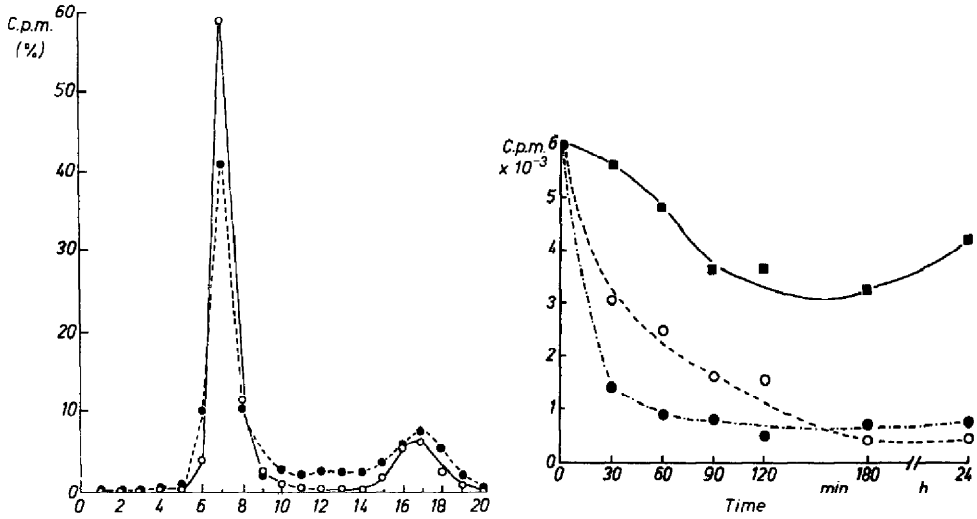

Fig. 6. Effect of disulfide-bond breaking reagent. Elution profile from a Bio-Gel A-50m column before (—○—○—) and after iodoacetamide treatment (--●--●--). For conditions see the Experimental section.

Fig. 7. The action of glycogen-degrading enzymes on the [^{14}C]glucan–protein described in Fig. 1. After incubating with phosphorylase *a* (--○--○--), soluble α-amylase (--●--●--), or insoluble α-amylase (—■—■—), as described in the Experimental section for the indicated times, the reaction was stopped by taking aliquots of 100μL each, spotting them on trichloroacetic acid-treated filter paper, and washing with trichloroacetic acid[25]. The radioactivity of the washed paper was measured in a scintillation spectrometer by adding a toluene-based scintillation fluid.

did the untreated material (Fig. 6), indicating that the high-molecular weight could not be attributed to glycogen–protein subunits held together by disulfide bonds. Enzymic treatment of the polysaccharide portion of the compound showed that the glucosidic chains may be degraded to a large extent by phosphorylase *a* and soluble α-amylase. On the other hand, degradation by insoluble α-amylase was more limited (Fig. 7).

After incubation with soluble α-amylase, the [^{14}C]glucose components were converted into two populations of products, as seen on Bio-Gel A-50m fractionation (Fig. 8). Most of the ^{14}C-labeled material was associated with the usual oligosaccharide products of glycogen α-amylolysis (Fig. 8, fractions 14–20). About 15% of the radioactivity was still in material of relatively high-molecular weight where protein (not α-amylase) was also present (fractions 6–13). Protein-free glycogen so degraded does not give rise to the higher-molecular-weight carbohydrate-containing fraction.

To sum up, the [^{14}C]glucan–protein complex synthesized *in vitro* by the rat liver enzyme preparations has the following properties: (a) The ^{14}C-labeled component behaves towards enzymes as does a (1→4)-α-D-glucan, being degraded by phosphorylase and by α-amylase. (b) The apparent molecular weight of the [^{14}C]glucan–protein is lowered when it is treated with glycogen-degrading enzymes (Figs. 7 and 8), or proteases (Figs. 2 and 3), or both. (c) When the glucan is de-

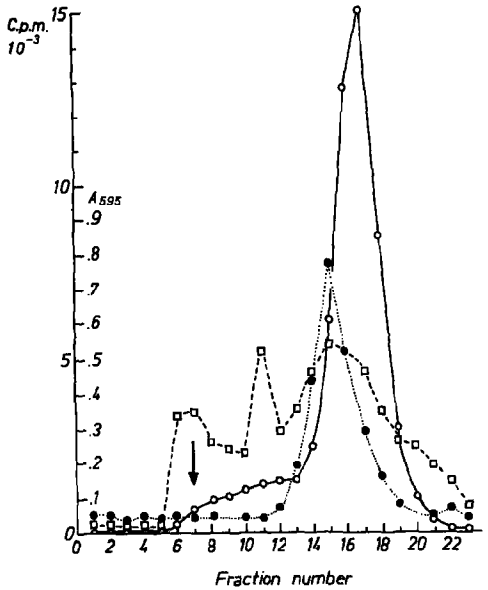

Fig. 8. Profile of elution from a Bio-Gel A-50m column after incubation of the [^{14}C]glucan–protein described in Fig. 1 with soluble α-amylase. After being incubated for 4 h at 37°, following the conditions described in the Experimental section, the mixture was passed through a Bio-Gel A-50m column (1 × 32 cm) and eluted with 100mM ammonium carbonate at pH 9.0. Fractions (0.5 mL each) were collected and the radioactivity (—○—○—) was measured on 100-μL aliquots. Aliquots from the same fractions were also taken for the determination of the protein content (--□--□--) by the Bradford method. In order to localize the position of α-amylase in the column (····●····●····), a solution of the enzyme was passed through and the protein content measured. The arrow indicates the void volume. The position of elution of undegraded glucan–protein may be seen in Fig. 3.

graded by α-amylase, a ^{14}C-labeled component is formed which is associated with protein and has a molecular weight higher than that of the usual oligosaccharides formed from glycogen by α-amylase (Fig. 8). (d) When the protein is degraded by proteases and the glucan by α-amylase, electrophoretically mobile compounds are formed which behave in the manner expected from oligosaccharide–peptides. (e) Glucan and protein remain together during NaDodSO$_4$-gel electrophoresis.

The foregoing properties of the glucan–protein, synthesized *in vitro*, are the same as those of a glycogen fraction from rabbit liver, insoluble in trichloroacetic acid[11]. The glycogen in the latter product was also accompanied by protein that behaved as if it were covalently bound and remained associated with the glycogen when it was submitted to similar fractionation procedures or when it was digested with glycogen- or protein-degrading enzymes. The same is true for rabbit-muscle glycogen, which contains covalently bound protein[12]. Similarly, a glucoprotein synthesized by a purified rat heart preparation, showed after PAGE–NaDodSO$_4$ analysis a (1→4)-[^{14}C]glucan covalently linked to protein[26].

We conclude that we were able to detect a similar, trichloroacetic acid-insoluble glucan fraction in rat liver by the process of labeling it with UDP-

[^{14}C]glucose by use of endogenous glycogen synthase, also present in the same 150 000*g* pellet[4]. By the several tests applied, the glucan is attached to protein with sufficient tenacity to suggest covalent bonding. A [^{14}C]glucose-containing fragment that behaves as a glycopeptide in electrophoresis could be derived from the glucose-labeled putative acceptor-protein after proteolysis and amylolysis. This result establishes the validity of our concept that the glucose-accepting protein is at the biogenetic origin of glycogen. It further has the practical advantage of demonstrating that the nature of the covalent linkage between a glucose residue and the accepting amino acid residue in the protein can now be elucidated by structural analysis of this fragment, in which the glycogen–protein linkage region has been concentrated. As presented by Goldsmith *et al.*[27], a glycogen molecule having a molecular weight of 10^7 has in its central part a very small protein core. Thus, because of the relatively small amount of acceptor protein that is likely to be present, it makes the structural analysis rather difficult. These results add support to our hypothesis that glycogen synthesis in liver is initiated on a protein primer[4,12,14,26]. The question is raised whether such initiation might be a general mechanism for the genesis of other polysaccharides.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Luis F. Leloir for continuous help and support and to the other members of the Instituto de Investigaciones Bioquimicas "Fundacion Campomar" as well as to Drs. Ernest Lee, James Aylward, and other members of the Department of Biochemistry from the University of Miami, School of Medicine, for helpful discussions and criticism. The skilful technical assistance of Ms. Susana Raffo in the preparation of labeled sugar nucleotides is gratefully acknowledged.

REFERENCES

1 W. J. WHELAN, *Trends Biochem. Sci.*, 1 (1976) 13–15.
2 C. R. KRISMAN, *Biochem. Biophys. Res. Commun.*, 46 (1972) 1206–1216.
3 C. R. KRISMAN, *Ann. N.Y. Acad. Sci.*, 210 (1973) 81–89.
4 C. R. KRISMAN AND R. BARENGO, *Eur. J. Biochem.*, 52 (1975) 117–123.
5 P. BULPIN, C. R. KRISMAN, AND W. J. WHELAN, in J. J. MARSHALL (Ed.), *Mechanisms of Saccharide Polymerization and Depolymerization*, Academic Press, New York, 1980, pp. 215–227.
6 R. BARENGO AND C. R. KRISMAN, *Biochim. Biophys. Acta*, 540 (1978) 190–196.
7 H. TAKAHARA AND K. MATSUDA, *J. Biochem. (Tokyo)*, 81 (1977) 1587–1594.
8 H. TAKAHARA AND K. MATSUDA, *J. Biochem. (Tokyo)*, 81 (1979) 907–914.
9 J. TANDECARZ, N. LAVINTMAN, AND C. E. CARDINI, *Biochim. Biophys. Acta*, 339 (1975) 345–355.
10 W. BARTLEY AND B. DEAN, *Anal. Biochem.*, 25 (1968) 99–108.
11 N. A. BUTLER, E. Y. C. LEE, AND W. J. WHELAN, *Carbohydr. Res.*, 55 (1977) 73–82.
12 L. D. KENNEDY, B. R. KIRKMAN, J. LOMAKO, I. RODRIGUEZ, AND W. J. WHELAN, in M. C. BERMAN, W. GEVERS, AND L. H. OPIE (Eds.), *Membranes and Muscle*, ICSO Press/IRL press, Oxford, 1985, pp. 65–84
13 J. A. CURTINO, H. J. MACCIONI, AND R. CAPUTTO, *J. Neurochem.*, 33 (1979) 139–147.
14 G. W. J. MATCHAM, N. B. PATIL, E. E. SMITH, AND W. J. WHELAN, in V. ESMANN, (Ed.), *Regulatory Mechanisms of Carbohydrate Metabolism*, Pergamon Press, Oxford, 1978, pp. 305–315.
15 J. A. THOMAS, K. K. SCHLENDER, AND J. LARNER, *Anal. Biochem.*, 25 (1968) 486–499.

16 S. SIEGEL, A. H. BRADY, AND W. M. AWAD, JR., *J. Biol. Chem.*, 247 (1972) 4155–4159.
17 M. R. BRADFORD, *Anal. Biochem.*, 72 (1976) 248–254.
18 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279–285.
19 R. G. SPIRO, *Methods Enzymol.*, 8 (1966) 26.
20 A. J. PARODI, C. R. KRISMAN, L. F. LELOIR, AND J. MORDOH, *Arch. Biochem. Biophys.*, 121 (1967) 769–778.
21 C. R. KRISMAN, *Anal. Biochem.*, 4 (1962) 17–23.
22 R. GEDDES, J. D. HARVEY, AND P. R. WILLIS, *Eur. J. Biochem.*, 81 (1977) 465–472.
23 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552–557.
24 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
25 C. R. KRISMAN AND R. BARENGO, *Anal. Biochem.*, 66 (1975) 584–589.
26 C. R. KRISMAN, R. BARENGO, M. L. BLUMENFELD, AND R. A. GEREMIA, (presentation dedicated to the memory of Renée Barengo) *Symp. Initiation Polysaccharide Biosyn., Abstr. Pan-Am. Biochem. Congr., IVth, (Symp. in memory of Carl Cori)*, (1984) 72.
27 E. GOLDSMITH, S. SPRANG, AND R. FLETTERICK, *J. Mol. Biol.*, 156 (1982) 411–427.