THE PRESENCE OF PHOSPHATE IN GLYCOGEN

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Received 29 August 1979
Revised version received 23 October 1979

1. Introduction

Despite the great amount of work carried out on glycogen structure there are several aspects which remain obscure. Glycogen isolated by mild methods has a very high molecular weight and appears to have labile bonds of unknown nature [1] and some –S–S– linkages [2]. Furthermore, protein is believed to form part of the glycogen molecule [3] and to be involved in the initiation of its biosynthesis [4,5]. Although other polysaccharides are known to contain bound phosphate, namely starch [6], glycogen is believed to be free from it. In fact, the lower the phosphate the purer the glycogen was considered to be [7–9].

This paper reports studies in which radioactive phosphate was administered to rats and moulds and then was found to follow glycogen during fractionation and degradation. The evidence is compatible with the presence of glycogen-bound phosphate.

2. Experimental

Adult Wistar rats were fasted for 4–5 days (water ad libitum). Thereafter each animal was fed through an intragastric canula with 2.5 ml of a solution containing 600 mg glucose and 100 mg casaminoacids plus 1–2 mCi of carrier-free $H_3{}^{32}PO_4$. In some experiments [^{14}C]glucose (250 mCi/mmol) was also administered. After 1 h the animals were allowed to eat standard laboratory chow and 2 h later they were sacrificed. The livers were homogenized in 3% $HgCl_2$ for glycogen isolation [8].

Neurospora crassa (St. Lawrence 74-A wild-type) cultures were grown as in [10] but the phosphate of the Vogel's medium was reduced to 1.85 mM. Labelled phosphate (5 mCi) was supplied at the beginning and at the late log phase of the 500 ml culture. The lyophilized mycelia were powdered in a Omni-Mixer before glycogen extraction with the $HgCl_2$ solution.

After repeated precipitations with ethanol and washings with chloroform–methanol–water (2:1:0 and 1:1:0.3, by vol.), glycogen fractionation was carried out with DEAE-cellulose [11].

The following methods were used: iodine for glycogen [12]; phenol–sulfuric for total hexoses [13], Lowry's [14] for proteins; molybdate for P_i [15] but with nitric acid replacing hydrogen peroxide in the oxidative step. Development of standard sugar phosphates in the electrophoretograms were carried out with molybdate spray [16].

^{32}P was measured by its Cerenkov radiation and the ^{14}C by scintillation counting with the Bray's solution.

3. Results

Labelled glycogens from rats and *Neurospora crassa* gave similar results when analyzed with the general methodology afterwards detailed, so that general comments are made indistinctly. In the illustrated experiments the glycogen source is inserted in the legend of the figures. *Neurospora* ^{32}P-labeled glycogen was selected for the experiments of fig.2,3 and table 1 in view of its higher specific activity.

3.1. *DEAE-cellulose fractionation*

Glycogen extracted with $HgCl_2$ and washed with lipid solvents still contains considerable amounts of

^{32}P. By chromatography on DEAE-cellulose several fractions can be separated as shown in fig.1. Most of the glycogen but very little [^{32}P]phosphate (peak I) was not retained by the column. Elution with increasing salt leads to the appearance of two peaks (II, III) which contain both glycogen and [^{32}P]phosphate. A last peak (IV) containing most of the [^{32}P]phosphate can be eluted with more salt but it contains very little glycogen. The percentages of glycogen in the eluted peaks were, respectively, 60, 29, 11 and 1% but these figures were variable in different experiments. The molecular weight of the glycogen in these peaks appears to be I < II < III as judged by the A_{520}. These results agree with those in [30]. As may be seen in fig.1 all the [^{14}C]glucose label coincides and is proportional to the glycogen content of each fraction.

^{32}P-labeled glycogen was found to bind not only to DEAE-cellulose but also to other anion exchangers (e.g., AE- and GE-celluloses, DEAE–Sephadex). Cation exchangers (e.g., CM-cellulose and CM–Sephadex) did not bind [^{32}P]glycogen.

The phosphate content (0.1–0.17%) of glycogen extracted with $HgCl_2$ was found to be somewhat lower than that reported in the original method [8] possibly due to the repeated organic solvent washings that remove part of it. Most of the phosphate was recovered in peak IV (eluted with ~0.5 M LiCl). Phosphate determination in the other peaks was very difficult due to the large amounts of organic matter

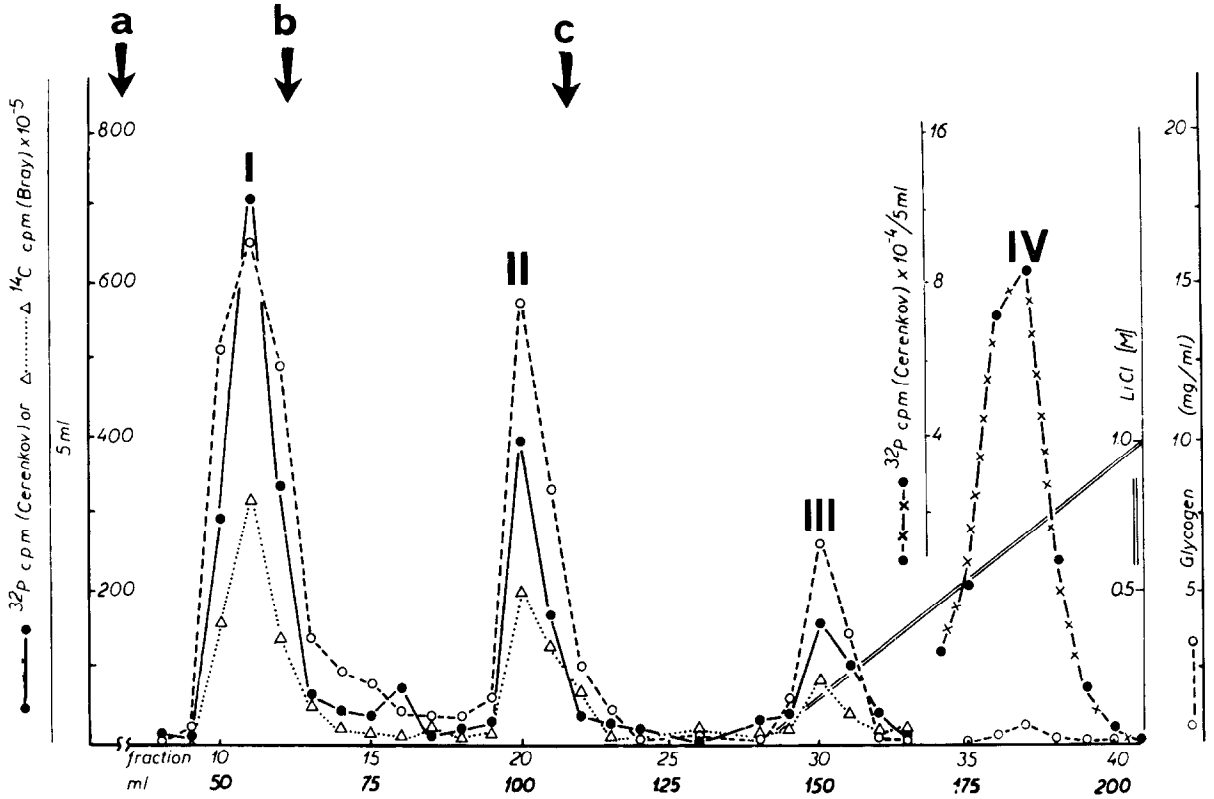

Fig.1. DEAE-cellulose chromatography of ^{32}P-labeled glycogen. Glycogen (~140 mg) was obtained from the liver of one rat pulsed with 1 mCi of $H_3{}^{32}PO_4$ plus 0.02 mCi [^{14}C]glucose. The extract was poured into a DEAE-cellulose column (Cl^- form; V_t = 60 ml) equilibrated with water. Elution was carried out by addition of water (a), 10 mM LiCl (b) and a gradient of LiCl 20–2000 mM where indicated by the arrows. Cl^- appeared in tube 20. The double line indicates the Cl^- concentration in the eluate. No correction was applied for the contribution of ^{32}P to the measured ^{14}C-radioactivity (Bray) because there was a difference of >300-times in the orders of magnitude. In the settings employed the ratio Bray/Cerenkov for ^{32}P was 2. Values of 4.6, 33.9 and 57.6 × 10^{-3} were obtained, respectively, for glycogens from peaks I, II and III when specific turbidity was expressed as the quotient between A_{520} unit and glycogen content (mg/ml).

to be processed. The values (0.1–0.18 mg/g) found for peaks II and III were higher than those of peak I (< 0.05 mg/g) suggesting that phosphorus is involved in the binding to DEAE-cellulose.

3.2. *Further studies on the DEAE-cellulose fractions*

Several procedures were used in order to find out if the [^{32}P]phosphate and glycogen could be separated:

3.2.1. Filtration on Bio-Gel A50m

When glycogen from peaks II and III was passed through a Bio Gel 50m column both the glycogen and [^{32}P]phosphate were eluted together before the Blue Dextran 2000 marker (av. mol. wt 2 × 10^6). Part of peak I sometimes appeared after this marker, but again ^{32}P-label coincided with glycogen (fig.5A). When saline, 6 M guanidine hydrochloride or 8 M urea/1% SDS were used as solvents the filtration profiles were the same as with water and glycogen and [^{32}P] phosphate always co-eluted. This would rule out an association of the ^{32}P-label to the high molecular weight glycogen through hydrogen bonding.

3.2.2. Affinity chromatography with Con A–Sepharose

Concanavalin A (con A) bound to Sepharose can be used for the purification of glycogen. The result of an experiment in which 1 M NaCl was included to avoid non-specific interactions is shown in fig.2. ^{32}P-labeled glycogen was bound to the immobilized lectin and most of it was eluted with methyl α-mannopyranoside. In the fractions eluted with 10 mM ligand the ^{32}P-label and glycogen coincided.

3.2.3. CsCl gradients

Since glycogen and protein have different densities, centrifugation in CsCl gradients was used in order to confirm the association of [^{32}P]phosphate to glycogen. Glycogen from peaks I, II and III banded very sharply after 48 h centrifugation at 30–35 × 10^3 rev./min.

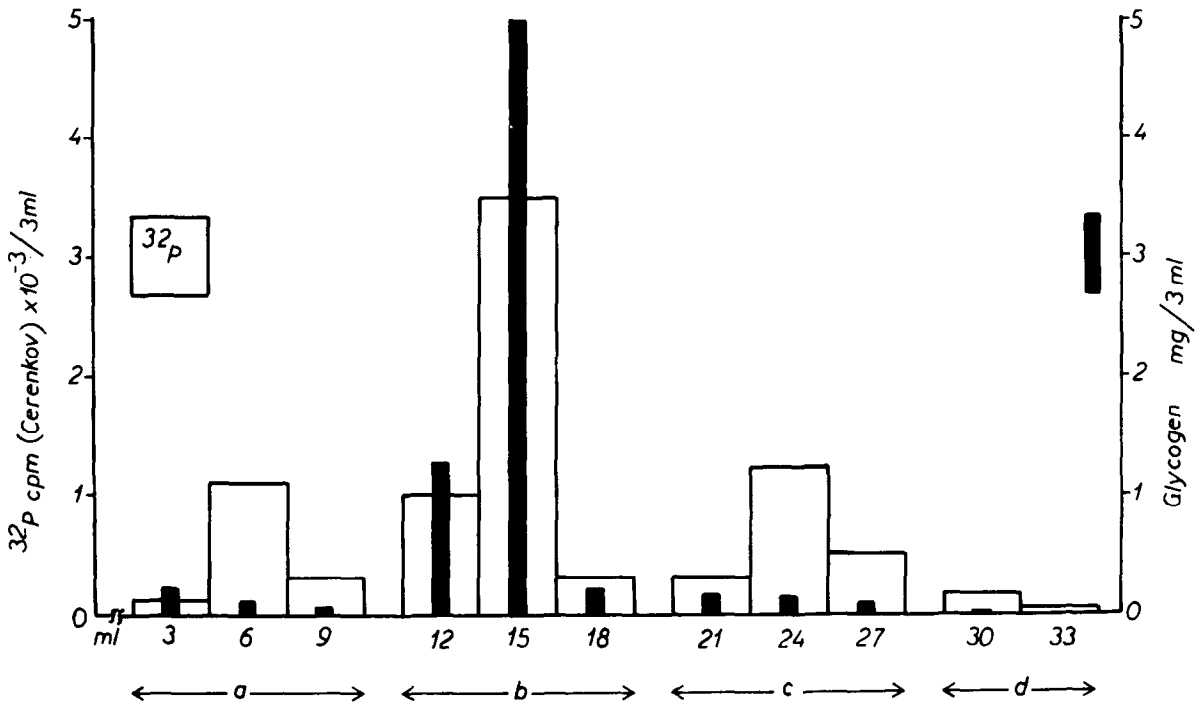

Fig.2. Affinity chromatography of ^{32}P-labeled glycogen on Con A–Sepharose. DEAE-cellulose peak I from *Neurospora* ^{32}P-labeled glycogen (~7.8 mg) was pre-filtered on Bio-Gel 50m and then poured into the affinity column (V_t = 3 ml; 3 ml fractions) equilibrated with buffer (a). (a) 100 mM HEPES–NaOH (pH 7.0) buffer containing 1 M NaCl, 0.1 mM DTT and 1 mM each of Mg, Mn and Ca chlorides. (b) 10 mM methyl α-mannopyranoside in buffer (a). (c) 50 mM methyl α-mannopyranoside in buffer (a). (d) 12.5% D-glucose in buffer (a). The fraction eluted with (b) had spec. act. 680 cpm ^{32}P/mg glycogen).

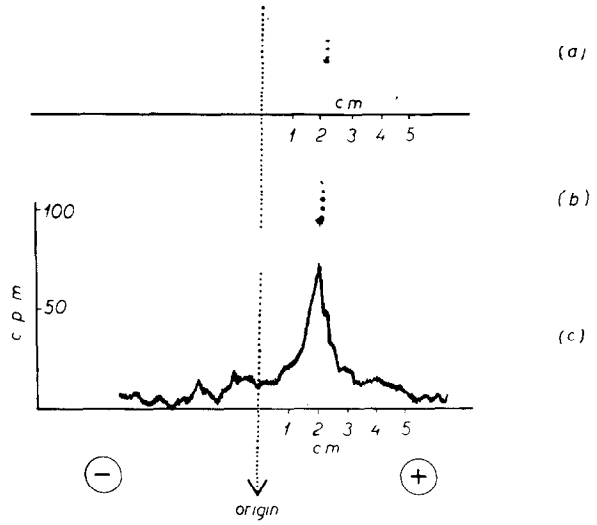

Fig.3. Cellulose acetate electrophoresis of Procion-dyed ^{32}P-labeled glycogen. Purified *Neurospora crassa* ^{32}P-labeled glycogen (fraction (b) from fig.2) was covalently stained with Procion-Blue GS [20] and freed of unreacted dye by Bio-Gel P-2 column chromatography. The cellulose acetate strip, pressed between two siliconized glass plates, was run in 100 mM borate (pH 9.3)/100 mM NaCl at 200 V for 25 min. (a) Photographic view of the transparentized strip. (b) ^{32}P-autoradiography of (a). (c) Radioscanning of (a).

No other noticeable ^{32}P-labeled fractions could be observed besides those coincident with the opalescent band of glycogen. An advantage of the high salt concentration of the CsCl solution is that it would dissociate any ^{32}P-bound contaminant joined through hydrogen bonds.

As seen in fig.1, peak IV contains very little glycogen and most of the ^{32}P-label. CsCl centrifugation of this fraction also yielded a discrete band of opalescent material, which was estimated as glycogen by colorimetry. However, the ^{32}P-radioactivity appeared spread out indicating the heterogeneity of the ^{32}P-labeled compounds. In fact, protein was easily detected in peak IV.

The average densities of ~1.7 g/cm^3 found for all ^{32}P-labeled glycogens are similar to those in [17,18].

3.2.4. Cellulose acetate electrophoresis of Procion-blue stained ^{32}P-labeled glycogen

Procion-blue reacts with hydroxyls due to its Cl-substituted triazinyl group [19]. As a result of the covalent attachment of the sulphonated chromophore residues, polysaccharides acquire negative charges and move to the anode during electrophoresis [20]. As shown in fig.3 after derivatization of purified ^{32}P-labeled glycogen with this dye, the ^{32}P-radioactivity was found to co-migrate with the blue-colored glycogen as detected by radioscanning and autoradiography.

3.2.5. Paper and glass fiber paper electrophoresis with borate buffers

Paper electrophoresis with borate buffers can be used for the separation of amylopolysaccharides [21]. Glycogen has relatively high mobility due to the presence of many non-reducing ends [22]. When ^{32}P-labeled glycogen was run by this method it was found that the ^{32}P-label migrated exactly with the glycogen. Whatman 3 MM paper permits the application of suitable amounts of sample in order to detect the glycogen with iodine vapours and the ^{32}P-label with radioscanning. It may be mentioned that ^{32}P-labeled glycogen extracted or treated with hot alkali retains a large part of the ^{32}P-radioactivity when examined by paper electrophoresis. The same is also true for phenol-extracted glycogen. In glass fiber paper electrophoresis the electroendosmotic effect is prevalent [21] and the net movement of both glycogen and the ^{32}P-label was towards the cathode.

3.3. *Enzymic treatments*

3.3.1. α-amylase

Prolonged treatment of purified ^{32}P-labeled glycogen with crystalline *B. subtilis* α-amylase yielded several [^{32}P]phosphorylated fractions as detected by column chromatography on Dowex-1-X4 (acetate form) eluted with increasing ammonium acetate solution. These were separated in preparative paper electrophoresis in pyridine acetate buffer. The major peak of the anion exchanger column (fig.4A) had an electrophoretic mobility similar to the standards of β-limit–α-phosphodextrins from starch [23] which basically consist of mono-(C_6)-phosphorylated maltotriose and maltotetraose. When this major ^{32}P-labeled component was treated with 0.7 N HCl at 100°C for 2 h, a treatment known to liberate glucose-6-phosphate from (C_6)-phosphorylated maltoligomers but not sufficient to dephosphorylate any of them [6], ^{32}P-containing compounds which migrate like the glucose-6-phosphate and orthophosphate standards were detected (fig.4B). The slower moving ^{32}P-labeled product now treated with glucose-

6-phosphate dehydrogenase gave rise to a faster moving ^{32}P-labeled compound (slightly faster than orthophosphate) which had the same mobility as a 6-phospho-gluconate standard (fig.4C). The esterification of hydroxyls other than (C_6) as reported for starch [6] would explain the liberation of free [^{32}P]phosphate under the acidic conditions used (fig.4B).

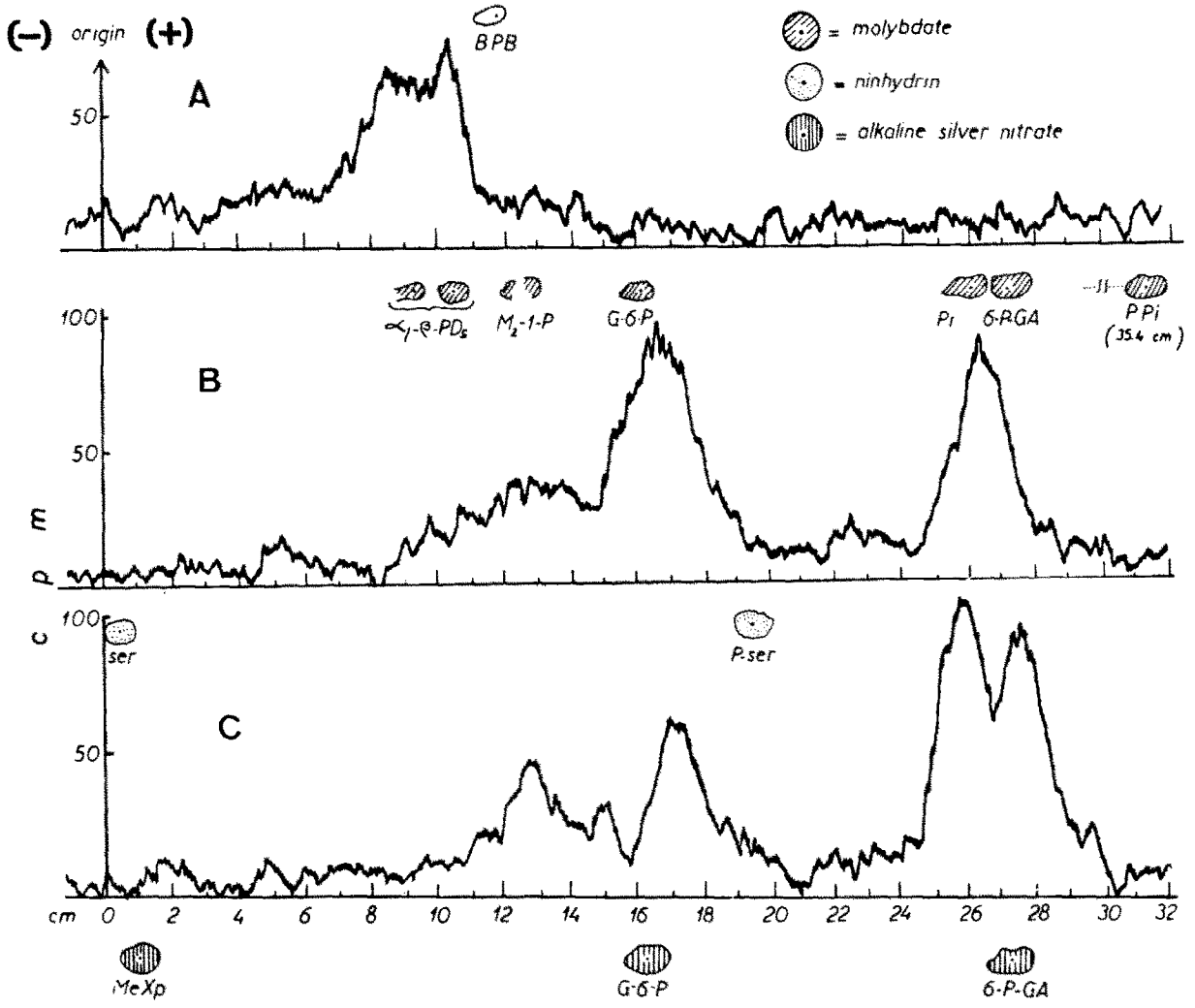

Fig.4. Paper electrophoresis of an α-amylolytic digest of [^{32}P]glycogen. Rat-liver ^{32}P-labeled glycogen (~50 mg) purified by DEAE-cellulose chromatography (peak I) and CsCl gradient was depolymerized with 350 units of 4 × crystalline *B. subtilis* α-amylase in 25 mM (pH 5.5) acetate buffer containing 0.1 mM $CaCl_2$ overnight at 35°C. The digest was poured into a Dowex-1-X4 column (acetate form: V_t = 3 ml) and eluted with stepwise increasing ammonium acetate solution. The major ^{32}P-labeled product was purified through preparative paper electrophoresis and again re-electrophoretized in the same system. Conditions: Whatman 3 MM paper (diluted acetic acid and EDTA washed); 10% pyridine–0.4% acetic acid (pH 6.5) buffer; CCl_4 coolant; 1.25 kV for 90 min. (A) Untreated major ^{32}P-product from α-amylolysis; (B) = (A) after 0.7 N HCl hydrolysis for 2 h at 100°C; (C) = (B) treated with 1 unit glucose-6-phosphate dehydrogenase (from *Torula* yeast) in 40 mM (pH 7.9) Tris–HCl buffer containing 10 mM oxidized NADP for 30 min at room temperature. In the top right side are indicated the reagents used for the standard development. *Abbreviations:* BPB, bromphenolblue; ser,P-ser, serine, phospho-serine; α_i-β-PD_s, mono-phospho-(C_6)-maltotetrahose and -maltotriose [23]; M_2-1-P, maltose-1-phsophate; G-6-P, glucose-6-phosphate; 6-P-GA, 6 phosphogluconic acid; P_i orthophosphate; PP_i, pyrophosphate; MeXp, methyl β-xylopyranoside.

3.3.2. Other enzymes

Treatment of ^{32}P-labeled glycogen with crystalline sweet-potato β-amylase followed by gel filtration showed that all ^{32}P-radioactivity remained in fractions of high molecular weight (fig.5). Treatment of ^{32}P-labeled glycogen with a mixture of α-amylase and *Rhizopus* amyloglucosidase resulted in a complete inclusion of the ^{32}P-label in the same gel.

The ineffectiveness of *E. coli* alkaline phosphatase to act upon a pool of the ^{32}P-labeled glycogens (< 10% of ^{32}P-label liberation without apparent change in the opalescence) points to the inaccessibility of the phosphate groups (the conversion of phosphothreonine to threonine was measured as control).

3.4. *Acid and alkaline treatments of intact ^{32}P-labeled glycogen*

As shown in table 1 (part B), treatment of ^{32}P-labeled glycogen with mild acid [24] liberates ^{32}P-radioactivity soluble in 75% ethanol. The nature of the ^{32}P-product(s) has not been determined. When the acid strength was raised 10 times (part C) < 50% of the ^{32}P-label was recovered in the precipitated glycogen. Appreciable amounts of the glycogen seem to be hydrolyzed as judged by the colorimetric method used here.

Paper electrophoresis (pyridine acetate buffer) was used to examine samples treated as above and also in others heated 60–90 min. Control ^{32}P-labeled glycogen remained always at the origin and very little [^{32}P]orthophosphate, if any, seemed to be liberated under any of these conditions, except for 0.1 N HCl beyond 20 min. The bulk of the ^{32}P-label moved progressively further from the origin but even with the stronger acid for 90 min its average migration was < 50% of that of the orthophosphate standard. In this extreme

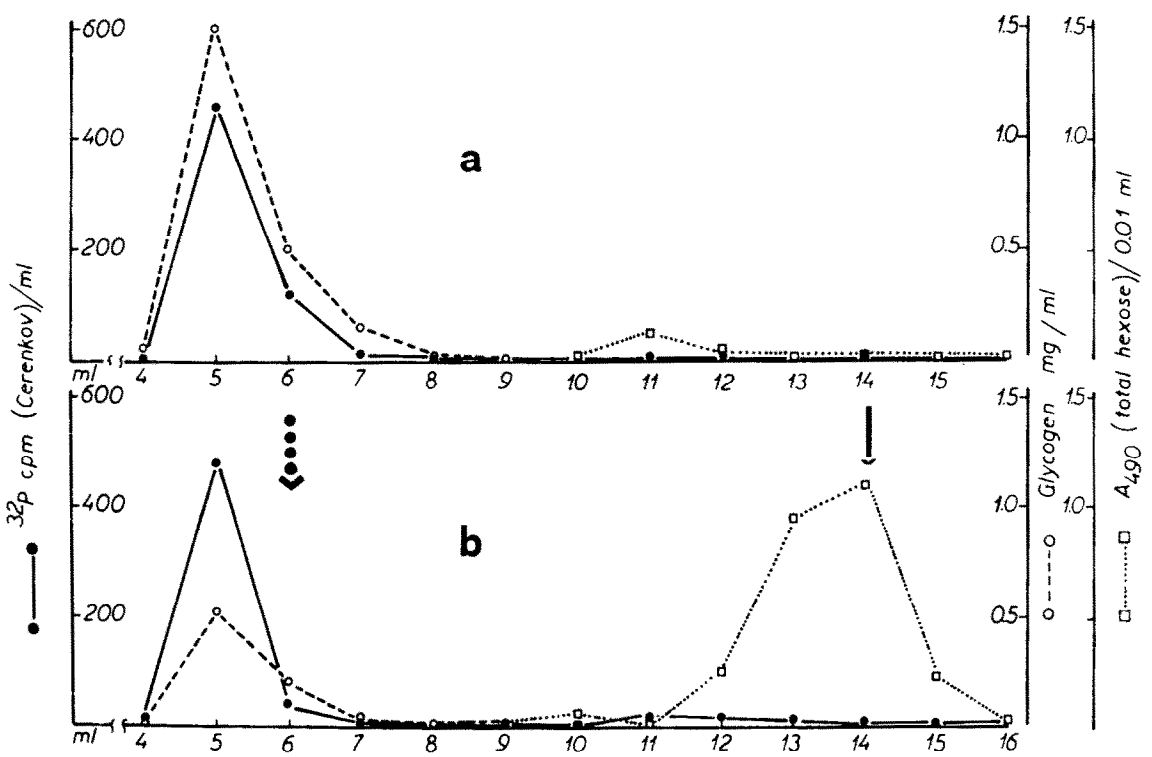

Fig.5. Gel filtration of ^{32}P-labeled glycogen on Bio-Gel 50m. (a) Rat liver ^{32}P-labeled glycogen (~1.5 mg) from DEAE-cellulose peak I (fig.1) run as control on a Bio-Gel 50m column (V_t = 12 ml; 1 ml fractions) equilibrated and eluted with water. (B) An equivalent amount of (a) pre-incubated with 8 units crystalline sweet-potato β-amylase in 100 mM acetate (pH 4.8) buffer for 4 h at 37°C. (The arrows ⋮ and ↓, respectively, indicate the elution volumes of the Blue-Dextran and maltose markers). (The same elution (a) pattern was observed with ^{32}P-labeled glycogens peaks II and III from DEAE-cellulose and also for runs in which 5 M guanidinine hydrochloride or 8 M urea/1% SDS were substituted for water as solvent.)

Table 1
Acid and alkaline lability of ^{32}P-labeled glycogen

	ethanol		
	pp 1.5	pp 3.0	Supern. 3.0 Vol.
No addition			
mg glycogen	1.8	0	0
cpm ^{32}P	940	5	75
Spec. act. (cpm/mg)	520	–	–
HCl 0.01 N			
mg glycogen	1.63[a]	0	0
cpm ^{32}P	440	20	570
Spec. act. (cpm/mg)	270	–	–
HCl 0.1 N			
mg glycogen	0.93	0.05	0
cpm ^{32}P	290	70	790
Spec. act. (cpm/mg)	312	–	–
KOH 5.88 N			
mg glycogen	1.83	0	0
cpm ^{32}P	650	40	560
Spec. act. (cpm/mg)	360	–	–

[a] 1.65 mg in another experiment

Neurospora ^{32}P-labeled glycogen from DEAE-cellulose and Bio-Gel 50m steps was precipitated with 1.0 vol. absolute ethanol from the concentrated 'milky' solution. Aliquots (1.82 mg–1150 cpm) were heated at 100°C for 15 min with the indicated additions. After cooling the supernatants from sequential precipitations with 1.5 and 3.0 vol. absolute ethanol were separated

case $\leqslant$ 10% of the total ^{32}P-radioactivity co-migrated with the orthophosphate standard.

This acid lability suggests the presence of linkages that are more labile than the α-1,4- and α-1,6-glycosidic linkages of glycogen.

Treatment under more drastic conditions (1.0 N HCl, 100°C, 60–90 min) liberated ~25% of the ^{32}P-label as a product that comigrated with the orthophosphate standard in the mentioned electrophoretic procedure. Glucose-6-phosphate, the more stable among the monophosphorylated glucoses, requires ~22 h to reach 50% hydrolysis under these conditions [25]. This is again indicative of the presence of [^{32}P]esthers other than the possible glucose-6-[^{32}P]-phosphate produced from the major ^{32}P-fragment of the amylolytic digest (fig.4A–C).

On the other hand, after a drastic alkaline treatment of ^{32}P-labeled glycogen the recovery of glycogen was complete (part D, table 1) but 50% of the ^{32}P-label was lost. This agrees with the occurrence of at least 2 different types of glycogen-bound [^{32}P]phosphate.

4. Discussion

Glycogen isolated from rats or *Neurospora crassa* fed with $H_3{}^{32}PO_4$ and purified by the DEAE-cellulose chromatography (following the $HgCl_2$ extraction and organic solvent washings) retained most of the ^{32}P-radioactivity as checked by several criteria. Thus the combined data are suggestive of a covalent linkage between ^{32}P and glycogen.

The presence of [^{32}P]phosphate in native glycogens liberated in part as glucose-6-[^{32}P]phosphate or making part of more labile linkages resembles the situation described for starch. Except for the excluded peak I from DEAE-cellulose chromatography the phosphate content of glycogen fractions is not very much lower than those described for a variety of vegetable starches [6], which range from 0.09–0.70 mg/g.

In the original method of DEAE-cellulose fractionation [11] the authors could find no protein or phosphorus in the glycogen to explain the adsorption on the anion exchanger, due presumably to the low con-

tent and the fact that the substances were unlabeled. Their explanation was based on the spacing of the DEAE-groups on the cellulose (50 Å) which would allow them to interact with each high particulated glycogen molecule (~1000 Å).

This explanation does not seem to hold since other anion exchangers also bind glycogen. The acid groups of glycogen are in part of phosphate but other anionic groups may also participate in this binding since a glycogen–peptide [3] from rat liver proved to be rich in aspartic and glutamic acids.

The results obtained by amylolytic depolymerization are suggestive of glucose-bound [^{32}P]phosphate(s) but the possibility that part of the ^{32}P could belong to phosphorylated amino acids cannot be ruled out since serine and theonine are prevalent amino acids in glycogen–peptide [3]. Furthermore, serine-phosphate is a usual component of glycogen synthetase and phosphorylase, enzymes closely associated with glycogen [9] and incidentally presenting acidic isoelectric points [26]. Interestingly glycogen synthetase from bacteria [27], though purified till homogeneity, has a very high content of polyglucose, an uncommon feature compared with classic glycoproteins in which a diversity of monosaccharides are present.

Finally, the proteo-glucan intermediary in the 'in vitro' synthesis of glycogen [4,5] is known to be very acid labile and (pyro)phosphate bridges were recently claimed to participate in the 'in vitro' synthesis of algal [28] and potato [29] saccharides.

This cumulative information deserves a more rigorous investigation concerning the possible involvement of phosphate in the linkage of glycogen to protein. The possible contribution of phosphate in the maintenance of the 'particulate' structure of glycogen is also of interest and some such studies are in progress.

Acknowledgements

Thanks are due to Dr L. F. Leloir for his interest and help with the preparation of this paper. I also thank Dr N. H. Torres for his encouragement and other members of the Instituto de Investigaciones Bioquímicas for helpful discussions. Dr E. Recondo provided the maltose-1-phosphate. The author is a Doctoral Fellow from Departamento de Bioquímica da U. F. Paraná, Curitiba, Brasil, and is supported by a fellowship from MEC/CAPES.

References

[1] Parodi, A. J., Krisman, C. R., Mordoh, J. and Leloir, L. F. (1967) Arch. Biochem. Biophys. 121, 769–778.
[2] Chee, N. P. and Geddes, R. (1977) FEBS Lett. 73, 164–166.
[3] Butler, N. A., Lee, E. Y. C. and Whelan, W. J. (1977) Carb. Res. 55, 73–82.
[4] Krisman, C. R. (1973) Ann. NY Acad. Sci. 210, 81–89.
[5] Takahara, H. and Matsuda, K. (1977) J. Biochem. 81, 1587–1594.
[6] Hizukuri, S., Tabata, S. and Nikuni, Z. (1970) Die Starke 22, 338–343.
[7] Northcote, D. H. (1954) Biochem. J. 58, 353–358.
[8] Mordoh, J., Krisman, C. R. and Leloir, L. F. (1966) Arch. Biochem. Biophys. 113, 265–272.
[9] Wanson, J. C. and Drochmans, P. (1968) J. Cell. Biol. 38, 130–150.
[10] Fontana, J. D. and Krisman, C. R. (1978) Biochem. Biophys. Acta 540, 183–189.
[11] Bobrova, L. N. and Stepanenko, B. N. (1972) Biokhimyia 37, 697–705.
[12] Krisman, C. R. (1962) Anal. Biochem. 4, 17–23.
[13] Dubois, M., Gilles, K. A., Hamilton, J. K., Rebbers, P. A. and Smith, F. (1956) Anal. Chem. 28, 350–356.
[14] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[15] Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468.
[16] Burrows, S., Grylls, F. S. M. and Harrison, J. S. (1950) Nature 170, 800–801.
[17] Aston, D. (1978) Endeavour (new ser.) 2, 142–148.
[18] Mandel, M., Schildkraudt, C. L. and Marmur, J. (1968) Methods Enzymol. 12B, 184–195.
[19] St. Groth, S. F., Webster, R. G. and Datyner, A. (1963) Biochim. Biophys. Acta 71, 377–391.
[20] Dudman, W. F. and Bishop, C. T. (1968) Can. J. Chem. 46, 3079–3084.
[21] Fuller, K. W. and Northcote, D. H. (1956) Biochem. J. 64, 657–663.
[22] Foster, A. B., Newton-Hearn, P. A. and Stacey, M. (1956) J. Chem. Soc. 30–36.
[23] Tabata, S., Hizukuri, S. and Nagat, K. (1978) Carb. Res. 67, 189–195.
[24] Pontis, H. G. and Leloir, L. F. (1972) in: Analytical Chemistry of Phosphorus Compounds (Halmann, M. ed) pp. 617–658.
[25] Peel, J. L. (1969) in: Data for Biochemical Research, 2nd edn, p. 110.
[26] Malamud, D. and Drysdale, J. W. (1978) Anal. Biochem. 86, 620–647.
[27] Fox, J., Kawaguchi, K., Greenberg, E. and Preiss, J. (1976) Biochemistry 15, 849–856.
[28] Tomos, A. D. and Northcote, D. H. (1978) Biochem. J. 174, 283–290.
[29] Tadecarz, J. S. and Cardini, C. E. (1979) Biochem. Biophys. Res. Commun. 86, 620–627.
[30] Bobrova, L. N. and Stepanenko, B. N. (1970) Dokl. Akad. Nauk SSSR, 191, 468–471.