REVISTA ESPAÑOLA DE FISIOLOGIA, 31, 163-172. 1975

Studies on Glycogen Metabolism in the Human Platelet. II. Metabolite Levels and Enzymes of the Glycogen Cycle *

J. Aguilar and M. Rosell-Pérez

Departamento de Bioquímica
Facultad de Farmacia
Barcelona - 14 (Spain)

(Received on 18 November, 1974)

J. AGUILAR and M. ROSELL-PEREZ. *Studies on Glycogen Metabolism in the Human Platelet. II. Metabolite Levels and Enzymes of the Glycogen Cycle.* Rev. esp. Fisiol., 31, 163-172. 1975.

The glycogen content of platelets and its degradation as response to energetic requirements are described. This degradation occurs in a rather rapid process which is active even when the cells are incubated at 4° C.

The levels of the enzymes responsible for the glycogen degradation, phosphorylase and amylo-1,6-glucosidase, analyzed in the same preparation agree with the scheme of glycogen depletion. Glycogen synthetase activities are also presented.

An increase in the glucose-6-P levels concomitant to an increase of the I (independent of glucose-6-P) activity of glycogen synthetase is presented. This increase is responsible for the high percentage of I activity in preparations obtained by freezing the cell suspension where the phosphatase of glycogen synthetase is inactivated.

The comparative analysis of I activities of crude extracts and their corresponding particulate fractions of 100,000 × *g*, where glucose-6-P is not present, together with experiments where glucose-6-P was removed either by column chromatography or enzymatic degradation, evince the identity of the sugar phosphate responsible for the high glycogen synthetase activity measured in absence of glucose-6-P.

Finally the possibility that glycogen degradation, in response to an energy demand, might increase the flow of its by-product glucose-1-P and the concentration of the intermediate glucose-6-P, is discussed.

Glycogen is a prominent feature of the platelets electron microscopy and a major component of these cells. Platelets actually contain as much glycogen as muscle tissue, accounting for a 30-50 % of the total blood glycogen content. The presence of the enzymes of the synthesis and degradation pathways of glycogen in platelets being now well documented, one can conclude that glycogen metabolism normally participates in the physiology of these cells.

* Work supported in part by a «Beca para la Formación de Personal Investigador» of the «Ministerio de Educación y Ciencia», a «Proyecto de Investigación de la Comisión Asesora de Investigación Científica y Técnica de la Presidencia del Gobierno» of Spain and a N.S.F. Grant GF-44115 from United States.

Blood platelets mostly rely on the glycogen storage when the twist of energy expenditure caused by their functions in the blood clotting and in the subsequent processes, the so called «Viscous metamorphosis» of the clot, occur.

In such a situation the blood carried supplies i.e. glucose, oxygen..., are not available to the cells trapped in the clot and consequently endogenous energy must support all energy dependent phenomena as shown by the decrease of glycogen content in physiological disadvantageous circumstances. Yet, as pointed out by SCOTT (22), under these high rates of glycogenolysis, the synthesis of the polysaccharide is not turned off but allowed to proceed, presumably at lower rates.

Degradation of glycogen proceeds in platelets through a reaction catalized by the enzyme phosphorylase (23), that cleaves α-1,4-glucosyl bonds, with the collaboration of the enzyme amylo-1,6-glucosidase (debranching enzyme), that cleaves α-1,6-glucosyl bond (15, 19).

Platelets phosphorylase seems to resemble muscle phosphorylase (31) as leucocyte phosphorylase seems to resemble the liver enzyme. The platelets enzyme appears to be in form of dimers or monomers, totally inactive, that can be converted to phosphorylase *a* or *b* and activated in the process (8-10, 22).

Synthesis of glycogen proceeds in platelets through the action of glycogen synthetase (11, 25, 28) that shows, in contrast to the enzyme of leukocytes (20), the two described forms (16, 17) as well as the set of primary regulatory enzymes that interconvert both forms (4, 18, 21).

We present in this paper some data concerning the relationship between synthesis and degradation of glycogen in human platelets and some possible explanation of the mechanism by which both processes seem to be active at the same time.

Materials and Methods

For the preparation of enzymes from platelets the intermediate fraction between the plasma and the red cells obtained in the plasmapheresis process was used as source of platelets. This fraction, enriched in leukocytes and platelets, was made up to 1/5 of its original volume with saline containing 0.001 M EDTA and centrifuged at $670 \times g$ during 10 minutes. The supernatant constitued the «platelet rich plasma» whose counting in an haemocytometer averaged 0.8 to 1.0×10^6 platelets/mm^3 and had a contamination of less that 4 red cells and 1 leukocyte per 10^4 platelets.

The platelet rich plasma was centrifuged at $3{,}000 \times g$ to sediment the cells and the sediment resuspended in 0.05 M Tris-0.001 M EDTA buffer (pH 7.8). The cell suspension was sonicated at 4° C for two succesive periods of 30 sec. Finally the cell debris was removed at $10{,}000 \times g$ for 20 minutes and the supernatant used as enzyme source, «crude extract».

The enzyme of the particulate fraction obtained by centrifugation of the crude extract at $100{,}000 \times g$ during 1 hour was also used in some experiments.

The differences in the preparation of the extracts for phosphorylase and amylo-1,6-glucosidase analysis will be described later.

UDP-glucose: α-1,4-glucan α-4-glucosyl transferase (glycogen synthetase) (E.C. 2.4.1.11) activity was measured as radioactivity incorporated into glycogen from ^{14}C-glucose-labeled UDPG as described by THOMAS *et al.* (27). The standard assay mixture contained 6.0×10^{-3} M UDPG (specific radioactivity 20,000 c.p.m./μmole), 1 % glycogen, 5.0×10^{-2} M Tris-5.0×10^{-3} M EDTA buffer (pH 7.8) and 1.0×10^{-2} M glucose-6-P when added. For the assay 30 μl of the enzyme source was added to 60 μl of the assay mixture and incubated at 30° C for 15 minutes. The incubation was terminated

by pipeting 75 μl of the reaction mixture on squares of filter paper Whatman 31 ET and immediately inmersed on 66 % ethanol. The papers containing the radioactive sample were thoroughly rinsed and then counted as described (27).

Phosphorylase activity was measured by the formation of Pi from glucose-1-P in presence of enzyme source. The standard assay mixture contained 0.032 M glucose-1-P, 0.05 M FNa, 0.01 M mercaptoethanol, 0.05 M Tris - 0.005 M EDTA buffer (pH 6.5) and 0.002 M AMP when added. For the assay 50 μl of enzyme source was added to 50 μl of the assay mixture and incubated at 30° C. The incubation was terminated by addition of 0.1 ml of 5 % trichloroacetic acid and Pi determined by the method of FISKE and SUBBAROW (3).

Amylo-1,6-glucosidase was measured from the incorporation of ^{14}C-glucose into glycogen in a reversed reaction (7). 0.4 ml of the reaction mixture containing: 3.0 μmole (0.3 μCi) of ^{14}C-glucose, 40 mg glycogen, 10 μmoles histidine chlorhydrate buffer (pH 6.5) and 10 mg of tissue were incubated at 37° C. The reaction was stopped by addition of 0.5 ml of 1.5 M trichloroacetic acid and 2 ml of distilled water. The glycogen was precipitated twice with 5 ml of 95 % ethanol. The precipitate was then digested in 2 ml of 20 % KOH at 100° C for 30 minutes; the glycogen in the alkaline digest reprecipitated thrice and finally resuspended in 0.7 ml of water.

The counting of radioactivity was accomplished by liquid scintillation in the following cocktail; 10 % naphtalene, 1 % 2,5-diphenyloxazol, 0.025 % 2,2-p-phenylen-bis-(5-phenyloxazol) and 20 % ethanol in dioxane.

The activity was expressed in units, the unit being the amount of enzyme per gram of tissue that incorporates 0.1 % of the counts added as radioactive glucose in 1 hour.

Glycogen determinations were made by the anthrone method (24). 5×10^9 cells were digested in 2 ml of 30 % KOH at 100° C for 4 hours. Glycogen was precipitated thrice in 66 % ethanol containing 1.5 % saturated Na_2SO_4 solution (6).

Glucose-6-P was measured following the appearance of NADPH in a reaction catalyzed by the enzyme glucose-6-P dehydrogenase. The reaction mixture contained: 30 μmoles of $MgCl_2$, 0.8 μmoles of NADP, 5 mg of glucose-6-P dehydrogenase (140 IU/mg) and the sample to be analyzed in a final volume of 0.3 ml of Tris 0.05 M EDTA - 0.001 M buffer (pH 7.8).

The use of an internal standard of glucose-6-P has allowed us to determine that 2 moles of NADP were reduced in our measurement per mole of glucose-6-P added. Consequently, to avoid the inespecific appearance of NADPH, the samples were rutinely boiled for 5 minutes and then centrifuged at $3,000 \times g$ before the measurement.

UDPG, 5'-AMP, Tris and glycogen (from rabbit liver) were purchased from Sigma Chemical Co. (St. Louis). ^{14}C-glucose and ^{14}C-glucose labeled UDPG were supplied by New England Nuclear Co. or by Radiochemical Center (Amersham). Glucose-6-P and glucose-6-P deshydrogenase were purchased from Boehringer (Mannheim); mercaptoethanol from Eastman Kodak Co.; ATP from Pabst Laboratories and anthrone from Merck.

Results

Glycogen content and its depletion. The cells obtained from plasmapheresis residues as described here showed a glycogen content of 8.0 ± 0.3 (mean $\pm$ S.E.) mg per 10^{10} platelets.

A rapid decline of this glycogen content can be observed when homogeneous aliquots of a cell suspension in saline containing 0.001 M EDTA were incubated at 4° C. The rate of decline was maximum between the eigth and the twenty fourth hour of incubation and the glyco-

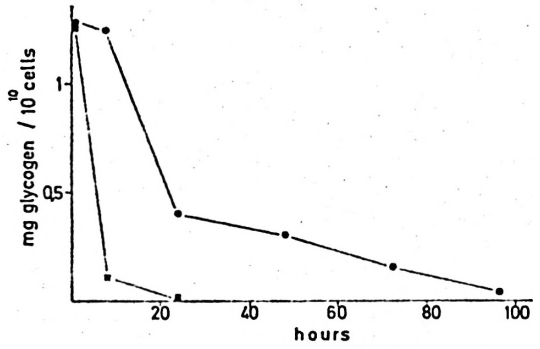

Fig. 1. *Depletion of glycogen in human platelets.*
Glycogen content is plotted versus time of preincubation of cell suspension. Glycogen was measured in aliquots of the platelet suspension as described in methods. Full circles (●) represent the lost of the glycogen in preparations preincubated at 4° C and full squares (■) the lost of glycogen when the preparation was preincubated at 20° C.

gen was almost completely depleted after 100 hours (fig. 1).

The same phenomena was observed when the incubation was carried out at 20° C, the level of glycogen being in this case 10 % of its original value after 8 hours of incubation and close to cero after 24 hours (fig. 1).

The levels of glycogen in the crude extract were analyzed in a set of experiments designed to study the stability of glycogen synthetase and phosphorylase with the incubation of the extract at 4° C. In these conditions we obtained a value of 250 mg of glycogen/ml of extract at cero time and a complete desappearance of the polysaccharide after 24 hours of incubation (not shown).

Phosphorylase activity. The phosphorylase activity of our cells analyzed under the conditions described in Methods was 0.05 μmoles/min/mg P when 5'-AMP was present in the assay and 0.045 μmoles/min/mg P in the absence of the activator (table I).

When the stability of the enzyme was to be checked in the extracts prepared for incubation at 4° C and in order to standardize the procedures, the enzyme was analyzed in Tris 0.05 M - EDTA 0.001 M buffer (pH 7.8) using the assay mixture described in Methods. The temperature of the assay was also changed, from 37° C to 30° C in these experiments. In such conditions the levels of activity were very similar to those described above and the decay after 48 hours of incubation at 4° C was only 10 % for the activity measured in the presence of 5'-AMP. The activity measured in absence of 5'-AMP seemed to be more affected by the conditions of the incubation process which promoted a 50 % loss of this activity (table I).

Amylo-1,6-glucosidase activity. The glycogen degradation process requires, in addition to the phosphorylase activity, the

Table I. *Phosphorylase activity in blood platelets*

Preparation of crude extract	Assay temperature °C	Specific activity in μmoles/min/mg P	
		—5'-AMP	+5'-AMP
Tris 0.05 M-EDTA 0.005 M-FNa 0.05 M-mercaptoethanol 0.01 M buffer (pH 6.5)	37	0.045	0.050
Tris 0.05 M-EDTA 0.001 M-mercaptoethanol 0.05 M buffer (pH 7.8)	30	0.033	0.046
Tris 0.05 M-EDTA 0.001 M-mercaptoethanol 0.05 M buffer (pH 7.8) after preincubation at 4° C for 48 hours	30	0.020	0.041

presence of a debranching activity accomplished by the enzyme amylo-1,6-glucosidase. We have been able to detect a debranching activity in the extracts of blood platelets obtained in our conditions. For the assay, a crude extract to a concentration of 100 mg of tissue per ml of Tris 0.05 M - EDTA 0.001 M (pH 7.8) was prepared according to the procedures described in Methods and immediately assayed. The reaction rate was linear for 60 minutes and the activity was calculated from the slope of the activity versus time plot. The value calculated for this activity under the conditions described was of 90 U/g of tissue.

Glycogen synthetase and its interconversion. The crude extract obtained in our conditions showed a glycogen synthetase specific activity of 5.9 ± 1.4 (mean $\pm$ S.E.) nmole/min/mg P for the total activity measured in presence of 0.01 M glucose-6-P. Both I (independent of glucose-6-P) and D (dependent of glucose 6-P) activities as described previously (16, 18) were detected and studied as well as the interconversion between them through the action of the corresponding primary regulatory enzymes (1).

When glycogen synthetase was analyzed at diferent times of incubation of the same cell suspension used for the studies of glycogen depletion above described, a fairly stable enzyme activity was observed during the first 8 hours followed by a constant decrease leading to a complete inactivation after 24 hours (not shown).

The enzyme from platelet extracts was totally converted to I form when preincubated at 30° C during 60 minutes in presence of 0.005 M mercaptoethanol reflecting a normally active glycogen synthetase phosphatase (1, 28) (table II).

If the preincubated extract was then centrifuged at 100,000 $\times$ *g*, the same percent of I activity was found, as expected, in the particulate fraction. However, if the crude extract was obtained by freezing and thawing instead of being obtained by sonication as described in Methods, a similar rate of D to I conversion was observed but the high percent of the I activity was mostly lost in the corresponding particulate fraction of 100,000 $\times$ *g* (table II).

This led us to the conclusion of glycogen synthetase phosphatase being inactivated by the freezing of cells, conclusion that was subsequently confirmed by the inactivation of the glycogen synthetase phosphatase activity observed when the crude extract was frozen or when aliquots of the same cell suspension were submitted to both, sonication and freezing, processes consecutively or viceversa.

Hence, the appearance of the I form activity in the extract treated by freezing temperatures, seemed not to be due to the conversion of the D to I of the glycogen synthetase but rather to the appearance of some indispensable factor, either for

Table II. *Glucose-6-P and glycogen synthetase I increase upon incubation*
The table shows the values for a preparation obtained by ultrasonic disruption and freezing and thawing of the cell suspension as well as the value for the corresponding particulate fraction of 100,000 $\times$ *g* resuspended in buffer.

	Preincubation time in minutes	Sonication		Freezing	
		%*	glucose-6-P mM	%	glucose-6-P mM
Crude extract	0	14	0.05	10	0.05
	60	80	0.10	41	0.24
	120	88	0.28	50	0.30
Particulate fraction of 100,000 $\times$ *g*	—	70	—	15	—

* (Activity-glucose-6-P/activity+glucose-6-P) $\times$ 100.

the D or the I form, which would not sediment at 100,000 × *g*.

Glucose-6-phosphate levels in platelets. In our search for an activating factor of glycogen synthetase that could be responsible for the phenomena described in the preceding paragraph, we started analyzing the glucose-6-P levels in our extracts and its possible variation upon incubation.

The glucose-6-P content of our extracts, measured according the procedures described in Methods, was 5×10^{-5} M, which in our conditions corresponds to 0.2 μmoles per 10^{11} cells.

When glucose-6-P levels were analyzed in crude extracts along the incubation that rose the I form activity, we found a parallel increase of the hexose phosphate concentration which reached levels 5 to 6 times higher after 90 minutes of incubation at 30° C. The same increase could be observed if the incubation was performed at 4° C during periods of time of 36 hours.

The increase in both glucose-6-P and I form activity was not affected by the method of homogenization used in the preparation of the extract.

We have to point out that only the addition of NADP to the extracts was required to detect its reduction suggesting that NADP was the limiting factor in the metabolism of glucose-6-P through the pentose phosphate pathway known to occur normally in platelets (30).

Identification of the activating factor of glycogen synthetase as glucose-6-P. The increase of glucose-6-P with the incubation of the extracts and the lost of I form activity in the particulate fraction of 100,000 × *g* of the extracts obtained by freezing of the cell suspension (table II), seemed to indicate that the I form activity in those extracts, lacking glycogen synthetase phosphatase, was an artifact caused by the glucose-6-P present in the enzyme sample which would activate the D form of glycogen synthetase in the I form assay.

Further evidence on the activation of glycogen synthetase by endogenously formed glucose-6-P was obtained in two types of experiments in which the glucose-6-P formed after preincubation was removed either by gel filtration or by treatment of the extracts with glucose-6-P dehydrogenase.

In a series of experiments an extract obtained by freezing of the cell suspension to inactivate the glycogen synthetase phosphatase was preincubated until 90 % of the total activity was in the I form, and then chromatographed in a column of Sephadex G-25. Fractions were collected an I and D activities of glycogen synthetase were analyzed. A peak of glycogen synthetase activity was obtained with a low percent (20 %) of I form activity (fig. 2) thus indicating that some component responsible for the high percent of the I form activity was missing in the fraction were the enzyme was present. Such component would be a small molecule, presumably glucose-6-P, that would not be expected to run with the enzyme in this gel.

Looking for a more specifiic method of removal of glucose-6-P from the system we tried its dehydrogenation by the enzymatic action of glucose-6-P dehydrogenase.

For this experiment the particulate fraction of 100,000 × *g* of aliquots of the same preincubated extract that had been obtained by freezing of the cell suspension were subsequently resuspended in buffer or in its supernatants as such or previously treated with glucose-6-P dehydrogenase. The dehydrogenase was allowed to react for 20 minutes at 30° C and the reaction mixture was then boiled for 5 minutes before the system was reconstituted, in order to inactivate the enzyme and avoid interferences with the glucose-6-P present in the assay of the D form activity.

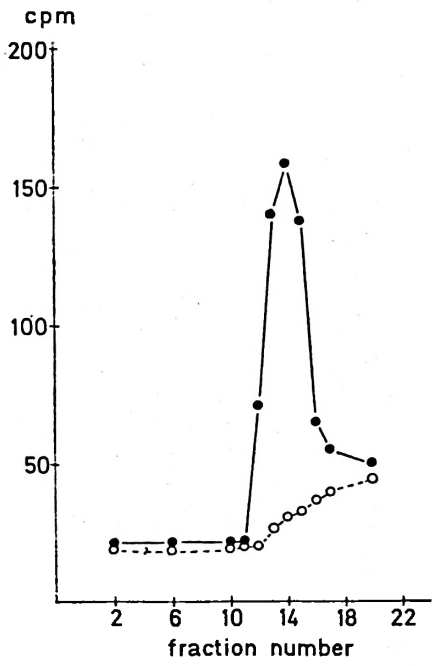

Fig. 2. *Sephadex G-25 chromatogram of glycogen synthetase extract obtained by freezing the cell suspension.*
An extract of human platelet suspension obtained by freezing and thawing of the cells was preincubated up to the point were 90 % of glycogen synthetase was independent of added glucose-6-P. The extract was then chromatographed on Sephadex G-25 and both glycogen synthetase activities measured in each fraction. A single peak of total activity was obtained and the corresponding percent of I activity was about 20 % in the fractions containing the enzymatic activity.

Though the results are masked by the high concentration of Mg^{++} required for the dehydrogenation which stimulates more the I form than the D form (1), a significant decrease in the percent of the I form activity was observed when the particulate fraction was resuspended in the supernatant treated in contrast to what happened when resuspended in the supernatant not treated (table III).

Discussion

The specific activities of glycogen synthetase and glycogen phosphorylase as well as the glycogen and glucose-6-P levels described here for platelets obtained from plasmapheresis residues are in the same range of the values reported by other investigators (11, 22, 25, 28, 31) for cells obtained by direct venipuncture, indicating the usefullness of such preparations. The amount of tissue easily obtainable by this method and the physiological conditions of the cells in it open many possibilities in the study of the biochemistry of platelets.

Amylo-1,6-glucosidase activity of blood platelets has not been to our knowledge previously reported and therefore can not be included in the data above compared. The specific activity obtained in our preparations is one half of the activity present in liver (7). Nevertheless, since the relationship phosphorylase/amylo-1,6-glu-

Table III. *Glucose-6-P removal by dehydrogenation*
The particulate fraction of 100,000 × *g* of a preincubated enzyme was resuspended in buffer, its own supernatant without any treatment or treated with glucose-6-P dehydrogenase.

Particulate fraction of 100,000×*g* resuspended in:	Activity in c.p.m.		Activity (—glucose-6-P/ +glucose-6-P) ×100
	—glucose-6-P	+glucose-6-P	
Buffer	60	590	10
Supernatant of 100,000×*g*	276	622	44
Supernatant treated with glucose-6-P dehydrogenase	104	506	20

cosidase is mantained in platelets, both activities having comparable lower values with respect to the liver activities, the regulation of glycogen degradation according to PALMER and RYMAN (15) can be supposed to work in platelets.

We have found an increase of about 6 fold in the glucose-6-P concentration with the preincubation of our extracts and a parallel increase in the I form activity of the glycogen synthetase which seems in fact to be due to the activation of the D form by the endogenous formed glucose-6-P. We have been able to show this phenomenon after having a preparation without glycogen synthetase phosphatase activity consequently without D to I form conversion, thus allowing us to detect the activation of the D form activity in the assay mixture without added glucose-6-P.

The glycogen synthetase phosphatase is known to be the most labile of the enzymes involved in the metabolism of glycogen and has only recently been purified (12, 26). Its sensitivity to freezing of extracts or cells has also been reported for the enzyme of leukocytes (13).

The possibility of the activation of the D form of glycogen synthetase by the glucose-6-P can be studied kinetically with the data obtained for this activation in a previous study (1). Accordingly, at the basal concentration of glucose-6-P, the D form of the enzyme is working at a 30% of its maximum velocity at saturating concentrations of substrate and activator. At 6 times higher concentration of glucose-6-P the enzyme would work at 66% of its maximum velocity and if the presence of Mg^{++} is considered, the cation lowering the K_a for glucose-6-P, these values would be 40% and 85% respectively of the above mentioned maximum velocity.

The increase in glucose-6-P concentration and its effect *in vivo* need more evidence since on the one hand the evaluation of the intracellular concentrations of metabolites is still very speculative and on the other hand, though the enzymes of the pathways metabolizing glucose-6-P, i.e.: glucose-6-phosphatase, glycolytic enzymes and pentose phosphate pathway enzymes are all soluble, a normal metabolism of the compound in our conditions cannot be assumed. Nevertheless, as an increased flow rate through the pathway seems to happen, as a consequence of the increased glycogenolysis, a net increase in the level of the key compound glucose-6-P can result.

The possibility of other activators besides glucose-6-P in the supernatant of 100,000 $\times$ *g* cannot be ruled out but our experiments show that a major decrease in the activation is obtained after removal of the hexose phosphate. It should be pointed out that gluconate-6-P, the product of the enzymatic dehydrogenation of glucose-6-P, shows only 1.7% of the activation of the D form by glucose-6-P (19).

The decrease in glycogen content upon incubation of the cells or extracts, the stability of glycogen phosphorylase and the inhibition of the D form activation by FNa, support the assumption of the glucose-1-P beeing formed in the glycogenolysis and subsequently transformed into glucose-6-P by phosphoglucomutase which happens to be inhibited by FNa (14).

As stated by GROSS *et al.* (5) a temporarily increase in the rate of formation of glycogen can be observed as an effect of thrombin and other platelet aggregating conditions and, therefore, glycogen synthesis as well as degradation processes, could be turned on simultaneously in a futile cycle like the one described by CLARK *et al.* (2) whose net flow would result in the accumulation or depletion of the polysaccharide.

The translation of these *in vitro* results to the *in vivo* facts is not yet possible and though a physiological meaning of these observations cannot be drawn out, the experiments are suggestive.

Resumen

En el presente trabajo se estudia el contenido en glucógeno de las plaquetas humanas y su degradación en respuesta a las necesidades energéticas de estas células. Dicho glucógeno se degrada en un proceso relativamente rápido, que resulta activo incluso cuando las células se incuban a 4° C.

Las actividades de los enzimas involucrados en la degradación del glucógeno, fosforilasa y amilo-1,6-glucosidasa, analizados en la misma preparación, parecen estar de acuerdo con el esquema de degradación del glucógeno. También se presentan las actividades de la glucógeno sintetasa.

Se describe un aumento en los niveles de glucosa-6-P concomitante con el incremento de la actividad glucógeno sintetasa I (independiente de glucosa-6-P) responsable del incremento de actividad medida en ausencia de glucosa-6-P, en preparaciones obtenidas por congelación de la suspensión celular en las que la fosfatasa de la glucógeno sintetasa se ha inactivado. Todo ello se pone de manifiesto por el análisis comparativo de los porcentajes de actividad I entre la fracción particulada de 100.000 × *g*, donde la glucosa-6-P no se halla presente, y los correspondientes extractos crudos.

Se ofrece mayor evidencia sobre la identificación de la glucosa-6-P como responsable de la elevada proporción de actividad I en experimentos en los que el activador se elimina por cromatografía o degradación enzimática.

Se discute, finalmente, la posibilidad de que la degradación de glucógeno en respuesta a un requerimiento energético, aumente el flujo en la vía de degradación de su producto glucosa-1-P y como consecuencia incremente la concentración del intermediario glucosa-6-P.

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