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Zonal distribution of glycogen synthesis in isolated rat hepatocytes

Josep M. Fernández-Novell[a], Carmen López-Iglesias[b], Juan C. Ferrer[a], Joan J. Guinovart[a,c,*]

[a] *Departament de Bioquímica i Biologia Molecular and Institut de Recerca Biomèdica de Barcelona-Parc Científic de Barcelona, Martí i Franquès 1, 7 planta, E-08028 Barcelona, Spain*
[b] *Serveis Científico-Tècnics, Universitat de Barcelona, E-08028 Barcelona, Spain*
[c] *Institut de Recerca Biomèdica de Barcelona-Parc Científic de Barcelona, Universitat de Barcelona, E-08028 Barcelona, Spain*

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Abstract Incubation of hepatocytes isolated from fasted rats with [^{14}C]glucose for short periods of time showed that the initial stages of glycogen synthesis occur near the plasma membrane. Incubation with [^{14}C]glucose followed by cold glucose demonstrated that glycogen synthesis is always active at the hepatocyte periphery and that previously synthesised glycogen moves towards the centre of the cell, while its place is filled by newly synthesised molecules. However, the reverse experiment, incubation with cold glucose before addition of [^{14}C]glucose, showed that, as glycogen synthesis progresses, it also becomes gradually active in more internal sites of the hepatocyte. These results indicate a spatial order in the synthesis of hepatic glycogen.
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Key words: Glycogen; Hepatocyte; Glycogen synthase; Electron microscopy

1. Introduction

Glycogen, a branched polymer of glucose, is found in most animal tissues and also in microbes. Its presence in such diverse life forms reflects the selective advantage conferred on the organisms by the ability to store excess glucose in a polymeric form. In mammals, the liver contains the highest concentration of glycogen. This polysaccharide is synthesised in hepatocytes when blood glucose levels are elevated, after a meal, and broken down during fasting periods when these levels are diminished, thereby contributing to the maintenance of glucose homeostasis.

Glycogen synthase (GS) catalyses the successive addition of glucose units through an α(1–4) bond to the non-reducing end of a growing glycogen molecule. Mammals express a specific liver isoform (LGS), the activity of which is in part controlled by the intracellular levels of glucose 6-phosphate, an allosteric activator and promoter of the covalent activation of GS through dephosphorylation [1]. Glucokinase (hexokinase IV) (GK) is responsible for the main glucose-phosphorylating activity in hepatocytes and shares the control of hepatic glycogen synthesis with GS [2].

Changes in the intracellular distribution of LGS [3,4] and GK [5–7] triggered by glucose correlate with stimulation of glycogen synthesis [8]. The translocation of these two enzymes suggests a spatial order in the metabolism of hepatic glycogen. Fluorescence microscopy of cultured hepatocytes has shown that the addition of glucose causes the translocation of LGS to the cell periphery and that the initial glycogen deposits are also formed near the plasma membrane of hepatocytes [4].

In early electron microscopy studies, which used osmium tetroxide fixation and staining of sections by uranium and lead salts, glycogen was described as 0.2–0.3 μm spheroid particles in muscle and rosettes composed of similar-sized particles in liver (for a review see [9]). Histochemical staining of glycogen, by a modification of the periodic acid Schiff (PAS) procedure introduced by Thièry [10], confirmed these findings. Subsequently, several studies have dealt with the morphology and ultrastructural localisation of glycogen deposits in liver [11,12] and muscle [13,14], but none has described the initial stages of glycogen synthesis in hepatocytes that contain very low amounts of the polysaccharide. Using [^{14}C]glucose as a precursor to radioactively label glycogen particles, here we describe the time course of the distribution of glycogenogenic sites in hepatocytes isolated from fasted rats.

2. Materials and methods

2.1. Animals and preparation of hepatocytes

Male Wistar rats weighing 200–230 g were used. Rats were deprived of food 24 h before the experiments, but had free access to drinking water. Suspensions of isolated parenchymal liver cells were prepared, as previously described [15]. Cells were resuspended in Krebs–Ringer–bicarbonate buffer (pH 7.4). Viability was checked by exclusion of trypan blue and preparations with viability below 90% were not used.

2.2. Culture conditions

Samples (4 ml) of these suspensions, containing 6–8×10^6 cells/ml, were incubated at 37°C with 30 mM glucose for 0 (control), 5, 10, 15, 20, and 30 min with gassing and continuous shaking (100 strokes/min). Cells were centrifuged at 3000×*g* for 20 s and washed twice with Krebs–Ringer–bicarbonate buffer. The cellular pellet was homogenised with 10 volumes of 30% KOH, boiled for 15 min and total glycogen content was measured after precipitation with 66% ethanol, as described [16]. Cells were also incubated with 30 mM [^{14}C]glucose (7.5 μCi/ml) for 30 min, centrifuged, washed and homogenised as above, and total radioactivity incorporated into the cells was measured using a scintillation counter. Glycogen was precipitated as above and radioactivity incorporated into the polysaccharide was also measured.

In another set of experiments, isolated hepatocytes were incubated with 30 mM [^{14}C]glucose (7.5 μCi/ml) for 5 or 10 min. Suspensions were then centrifuged at 3000×*g* for 20 s, the pellets were immediately washed with Krebs–Ringer–bicarbonate buffer to remove the

*Corresponding author. Fax: (34)-93-402 1219.
E-mail address: guinovart@pcb.ub.es (J.J. Guinovart).

Abbreviations: GS, glycogen synthase; LGS, liver glycogen synthase; GK, glucokinase; PAS, periodic acid Schiff

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radioisotope and cells were then incubated with 30 mM glucose for 5, 10, 15 or 20 min. In addition, hepatocytes were first incubated with 30 mM glucose for 10 or 15 min and then with 30 mM [^{14}C]glucose (7.5 μCi/ml) for another 5 or 10 min. At the end of the incubations, aliquots were prepared for microscopic localisation of radioactive glycogen.

2.3. Electron microscopy

Hepatocytes were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. They were then washed in 0.1 M phosphate buffer, dehydrated with graded acetone solutions and embedded in Spurr's resin. Postfixation with 1% osmium tetroxide was used for non-radioactive samples, but not for the radioactive ones because it is known to fade latent images in autoradiography. Ultrathin sections (70 nm thick) were obtained from each experiment using a Leica Ultracut ultramicrotome and deposited over Formvar-carbonated copper grids. Sections from radioactive samples were coated with a monolayer of Ilford L4 nuclear emulsion diluted 1:4 with distilled water using a tungsten wire loop and following the 'loop interference' technique [17]. After an exposure of 8 months, the silver grains were revealed with Phenidon. All sections were contrasted with uranyl acetate and lead citrate and examined in a Hitachi MT 800 electron microscope at 75 kV. The electron micrographs were analysed using the IMAT software (developed in the Scientific and Technical Services of the University of Barcelona). To correct the position of radioactive origin, taking into account the possible deviation of radiation, a 'probability circle' was drawn around every silver grain. Each cell was then divided into four radial zones of identical thickness between the plasma and the nuclear membranes, and a given silver grain was counted as belonging to one of the four radial zones when more than 75% of the area of its 'probability circle' was within the limits of this zone. The percentage of silver grains in each of the four radial zones was then calculated separately for every cell analysed and the results were expressed as the mean percentage ± S.E.M. of 15–20 independent determinations for every condition.

2.4. Materials

Glucose was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and [^{14}C]glucose from NEN (Boston, MA, USA). Analytical-grade reagents were purchased from Merck (Barcelona, Spain).

3. Results

3.1. Ultrastructural localisation of glycogen deposits in isolated hepatocytes

Isolated hepatocytes from fasted rats were incubated with 30 mM glucose for increasing periods of time up to 30 min. Total glycogen content increased gradually from 0.6 mg/g of wet weight in control (0 min) cells to 4 mg/g in the hepatocytes treated with 30 mM glucose for 30 min (Table 1), which is consistent with previously published results [18].

Aliquots of the control cells and those incubated with glucose for 30 min were also fixed, embedded in resin, treated with osmium tetroxide and finally contrasted with uranyl acetate and lead citrate. Electron microscopy showed very few glycogen particles in control hepatocytes. These particles were small and were always found near the cytoplasmic membrane (Fig. 1a). After a 30 min incubation with 30 mM glucose, a large increase in the number and the size of the particles was observed (Fig. 1b). They were found scattered throughout the cytoplasmic area, but concentrated in the proximity of the smooth endoplasmic reticulum cisternae (Fig. 1c). In the hepatocytes treated with glucose, glycogen labelled by a modification of the PAS procedure [10] was also found throughout the cytoplasm (data not shown).

3.2. Glycogen is initially synthesised near the plasma membrane

To identify the locus where initial glycogen synthesis takes place, we used [^{14}C]glucose to radioactively label nascent glycogen particles. First, we measured the amount of radioactivity incorporated into distinct fractions after incubation of isolated hepatocytes with 30 mM [^{14}C]glucose (7.5 μCi/ml) for 30 min Of the total radioactivity recovered from these cells (0.142 ± 0.005 μCi/ml), which corresponds to approximately 2% of that present in the medium, more than 80% (0.117 ± 0.005 μCi/ml) was incorporated into glycogen. This finding indicates that microscopic localisation of the radioactivity incorporated after short treatments with [^{14}C]glucose is an adequate method to establish the subcellular distribution of newly synthesised glycogen.

Hepatocytes were incubated with 30 mM [^{14}C]glucose (7.5 μCi/ml) for 5, 15 or 30 min and processed to detect radioactive glycogen by microscopic observation. In the high-resolution autoradiographies, which were obtained after exposures of about 8 months, the hepatocytes showed a well-preserved ultrastructure (Fig. 2). After a 5 min incubation with [^{14}C]glucose, the number of labelled glycogen particles, which appeared as electron-dense silver grains, was very low. However, radioactivity was clearly localised in close proximity to the plasma membrane (Fig. 2a). The content of radioactive label gradually increased with incubation time and after 20 min the electron-dense spots were all located in a crown, which spanned from the plasma membrane to more interior locations of the hepatocyte (Fig. 2b). Finally, in the hepatocytes treated with 30 mM [^{14}C]glucose for 30 min, the silver grains were abundant and were scattered throughout the cytoplasm (Fig. 2c).

To quantify the distribution of radioactively labelled glycogen in the interior of the hepatocytes, the cells were arbitrarily divided into four radial zones of identical thickness between the plasma and the nuclear membranes. The first zone was the most peripheral and included the plasma membrane, while the fourth was closest to the nuclear envelope. In hepatocytes treated with 30 mM [^{14}C]glucose for 5 min, the radioactive label was almost exclusively (90%) in the most exterior ring, with a minor percentage in the second ring (10%) (Fig. 2d). As the incubation proceeded, the interior rings gradually contained more labelled particles (Fig. 2e), and after 30 min, the four zones contained approximately equivalent numbers of silver grains (Fig. 2f). These observations indicate that when isolated hepatocytes with initially low glycogen content are incubated with glucose, new glycogen is synthesised only at the periphery of the hepatocyte, in close proximity to the

Table 1
Hepatocytes isolated from fasted rats were incubated with 30 mM glucose for a range of times

	Incubation time with 30 mM glucose (min)					
	0	5	10	15	20	30
Glycogen content (mg/g w.w.)	0.6 ± 0.1	0.7 ± 0.2	1.0 ± 0.2	1.3 ± 0.2	1.8 ± 0.3	4.0 ± 0.4

Glycogen content was measured as described in Section 2. Results are means ± S.D. for six to eight independent experiments.

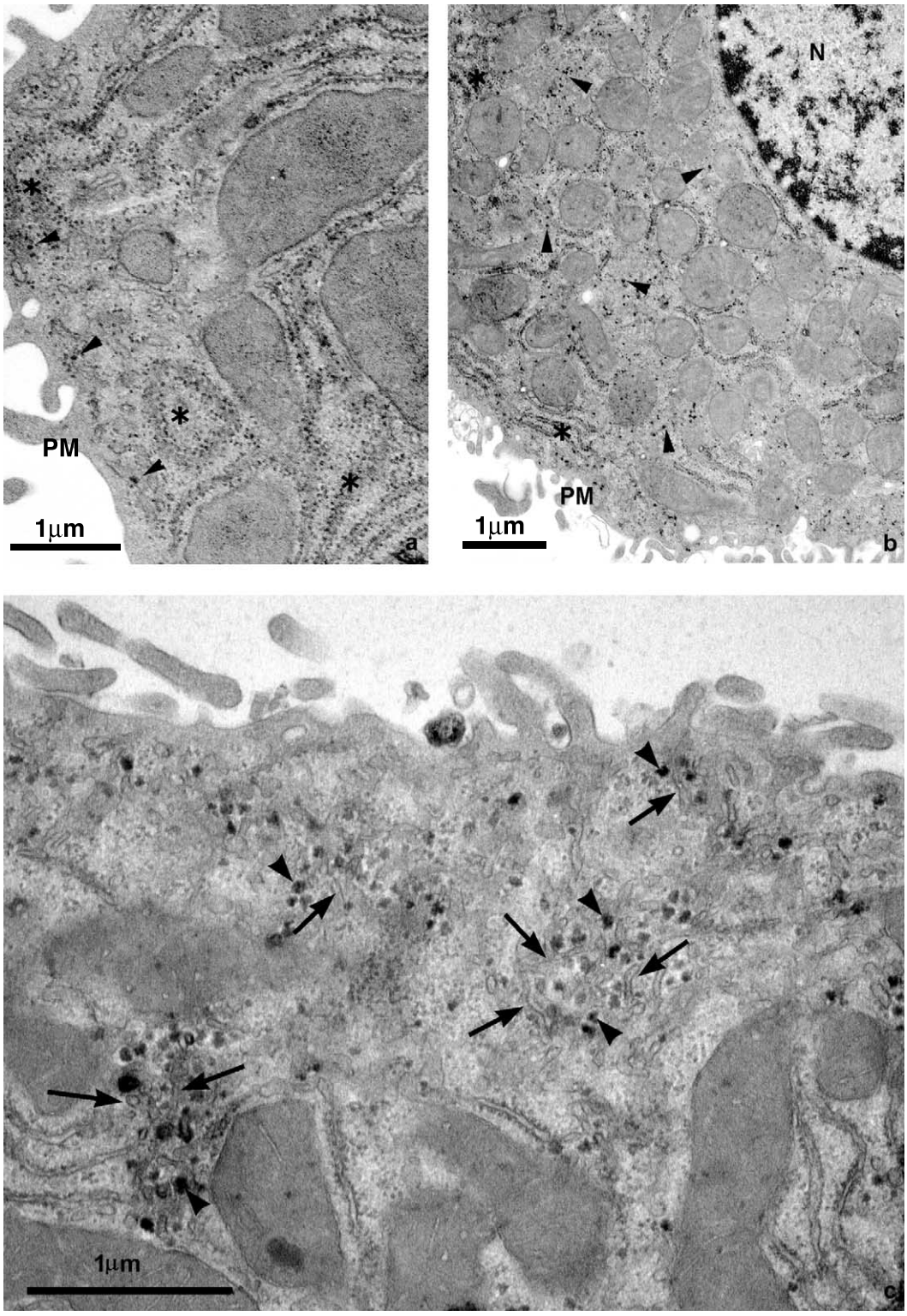

Fig. 1. Electron microscopy images of hepatocytes isolated from fasted rats after uranyl acetate and lead citrate staining. a: Hepatocyte showing a very small number of glycogen particles (arrowheads) located in the periphery of the cell, near the plasma membrane. b: Hepatocyte incubated for 30 min with 30 mM glucose showing a large number of glycogen particles (arrowheads) distributed throughout the cytoplasm. Rough endoplasmic reticulum zones showing cisternae with attached ribosomes and free ribosomes are labelled with stars. c: Detail of the cytoplasm of a hepatocyte after a 30 min incubation with 30 mM glucose. Glycogen particles (arrowheads) are found in the proximity of smooth endoplasmic reticulum cisternae (arrows). N: nucleus; PM: plasma membrane.

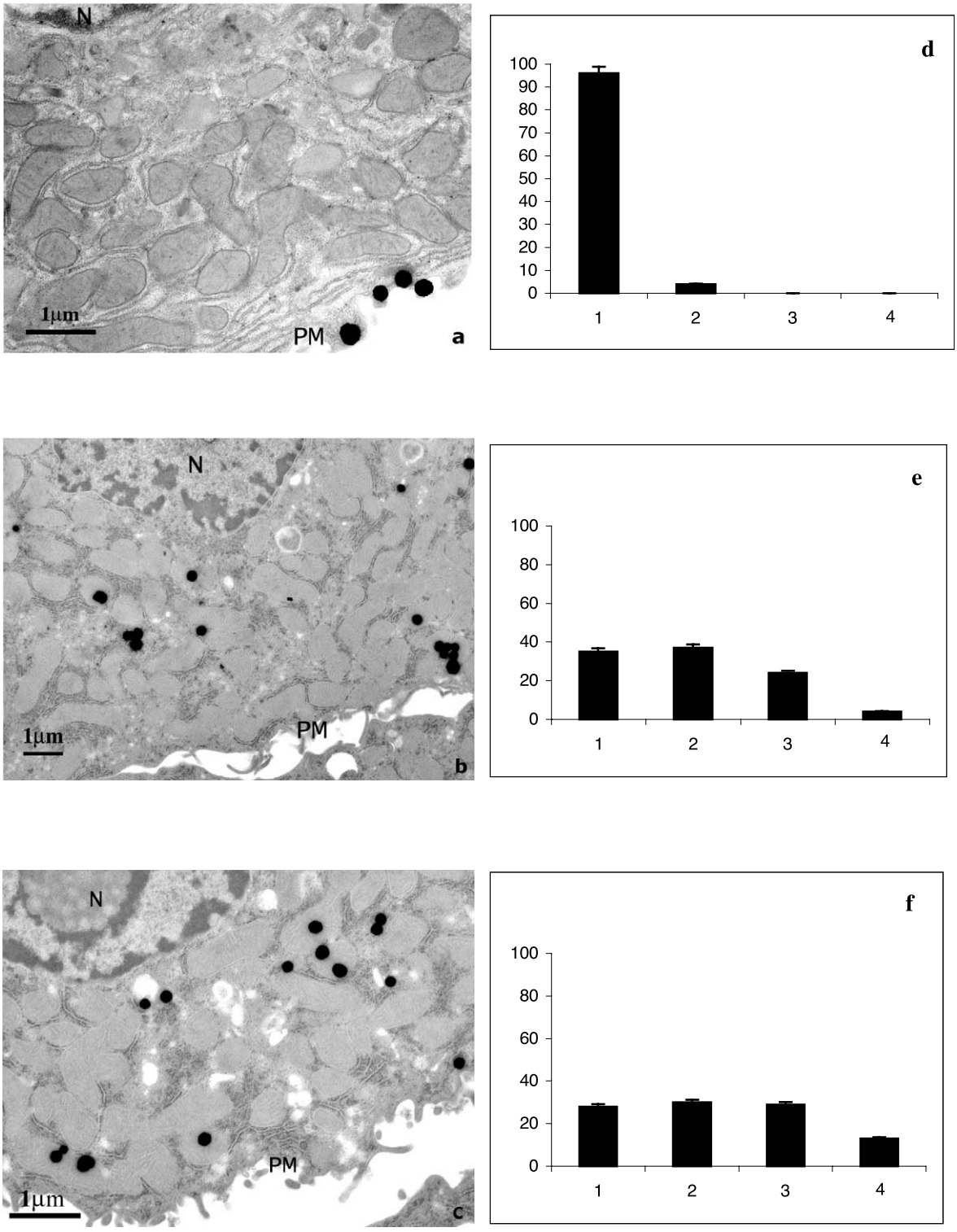

Fig. 2. Distribution of glycogen labelling in hepatocytes isolated from fasted rats treated with [^{14}C]glucose. Electron microscopy autoradiographies of ultrathin sections of hepatocytes incubated with 30 mM [^{14}C]glucose (7.5 μCi/ml) for 5 (a), 20 (b) or 30 min (c), as described in Section 2. Panels c–e are histograms that show the distribution of the radioactive label among four radial zones of identical thickness between the plasma membrane and the nucleus of hepatocytes incubated as in a–c, respectively. Results are mean ± S.E.M. of 15–20 determinations for each incubation. N: nucleus; PM: plasma membrane.

plasma membrane. Thereafter, glycogen deposits grow from the periphery towards the interior of the cell forming a crown that becomes thicker as the incubation time with glucose increases.

3.3. *Glycogen synthesis is always active near the plasma membrane*

The observations described here can be explained by two possible mechanisms, or a combination of both. In the first,

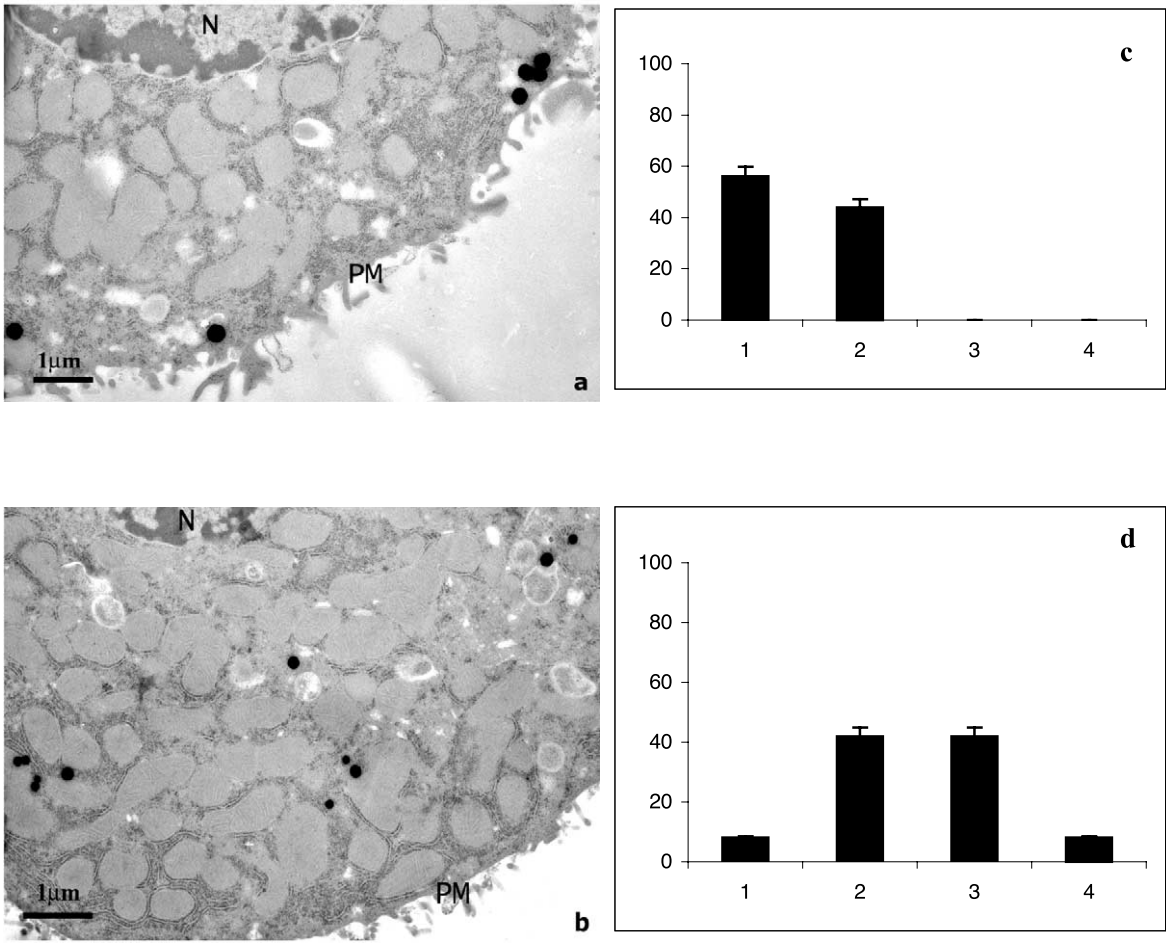

Fig. 3. Distribution of glycogen labelling in hepatocytes isolated from fasted rats after treatment with [^{14}C]glucose followed by non-radioactive glucose. Electron microscopy autoradiographies of ultrathin sections of hepatocytes first incubated with 30 mM [^{14}C]glucose (7.5 μCi/ml) and then with 30 mM non-radioactive glucose for 5*+5 min (a) or 10*+10 min (b). Panels c and d are histograms that show the distribution of the radioactive label in hepatocytes incubated as in a and b, respectively. Results are mean ± S.E.M. of 15–20 determinations for each incubation. N: nucleus; PM: plasma membrane.

glycogen may be exclusively synthesised near the plasma membrane while pre-existing molecules move towards the interior of the hepatocyte. Alternatively, the glycogen synthetic machinery could start working at the periphery of the cell and gradually move to interior locations as glycogen deposits grow. To differentiate between these two mechanisms, we performed pulse-chase experiments. Isolated hepatocytes were first incubated with 30 mM [^{14}C]glucose (7.5 μCi/ml) for 5*, 10*, 15* or 20* min and then with non-radioactive 30 mM glucose for another 5, 10 or 20 min. After a 5*+5 min incubation, glycogen labelling occurred mainly at the cell periphery (Fig. 3a), but the localisation of the silver grains was clearly more interior than after only 5 min of incubation with radioactive glucose (Fig. 2a). This internal localisation of the radioactive label was more evident in cells incubated for 10*+10 min, which showed a ring of electron-dense spots located between the plasma membrane and the nuclear envelope (Fig. 3b). Quantification of the number of silver grains in each concentric ring showed that after a 5*+5 min incubation approximately 55% and 45% of the label was found in the first and second zones, respectively (Fig. 3c). In the 10*+10 min incubations, the silver grains were mainly located in the second (45%) and third (45%) rings, and the remaining 10% was distributed between the most interior and exterior zones (Fig. 3d). Glycogen labelling showed intermediate positions between the periphery and the nucleus in other incubation times tested in the pulse-chase experiments (5*+10, 5*+15, 10*+15, 10*+20 min). The longer the incubation with unlabelled glucose the more interior the location of label (data not shown). These results indicate that, at least while there is net accumulation of glycogen, the synthesis of the polysaccharide is always active near the plasma membrane. Previously synthesised glycogen moves towards the centre of the cell and newly synthesised molecules fill its place.

3.4. *After initial stages glycogen synthesis becomes active at internal sites*

The previous observations, however, do not exclude the possibility that glycogen synthesis may also be active in other sites of the hepatocyte after initial synthesis and in the presence of a substantial amount of the polysaccharide. To test this hypothesis, hepatocytes were first incubated for 10 or 15 min with 30 mM non-radioactive glucose and then with 30 mM [^{14}C]glucose (7.5 μCi/ml) for 5* or 10* min, respectively. In both cases, radioactive glycogen was detected in a cytoplasmic area that extended from the cell periphery, near the plasma membrane, to more interior regions of the cells. After a 10+5* min incubation, silver grains were mainly distributed

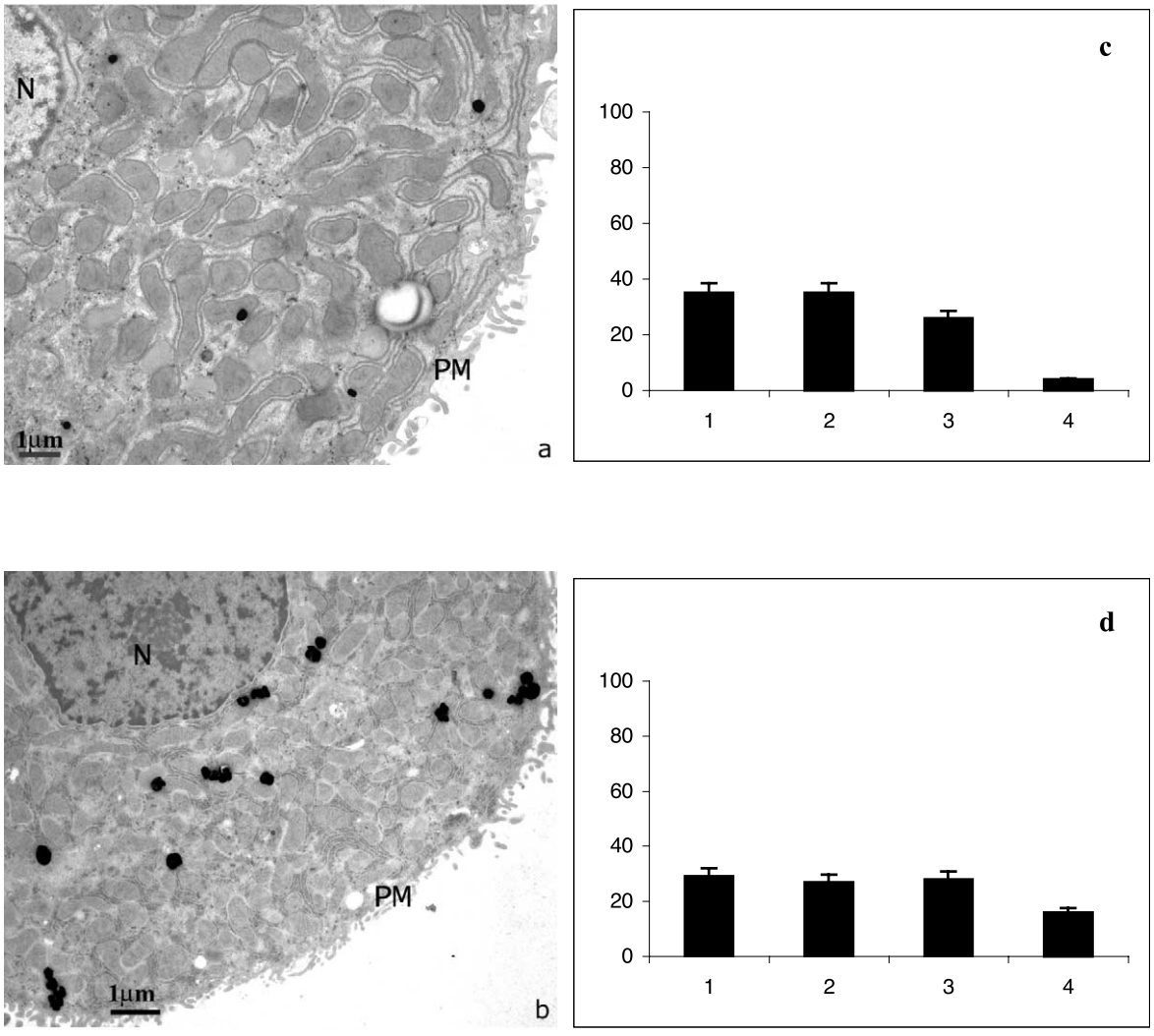

Fig. 4. Distribution of glycogen labelling in hepatocytes isolated from fasted rats after treatment with non-radioactive glucose followed by [^{14}C]glucose. Electron microscopy autoradiographies of ultrathin sections of hepatocytes first incubated with 30 mM non-radioactive glucose and then with 30 mM [^{14}C]glucose (7.5 μCi/ml) for 10+5* min (a) or 15+10* min (b). Panels c and d are histograms that show the distribution of the radioactive label in hepatocytes incubated as in a and b, respectively. Results are mean ± S.E.M. of 15–20 determinations for each incubation. N: nucleus; PM: plasma membrane.

among the three most external rings (Fig. 4a,c). In the 15+10* min incubation, the four zones presented almost identical amounts of radioactivity incorporated into glycogen (Fig. 4b,d). These observations are compatible only with glycogen synthesis becoming active at internal sites of the hepatocyte, in addition to the region close to the plasma membrane, as the synthesis of the polysaccharide progresses.

4. Discussion

Several studies have addressed the ultrastructural localisation of glycogen particles in muscle and liver tissues (for reviews see [9,14]). However, in the present report we describe the time course of the intracellular distribution of glycogen synthesis in isolated hepatocytes from fasted rats, which initially contain very low amounts of the polysaccharide.

The electron micrographs of osmium tetroxide-fixed hepatocytes corroborate previous observations that indicate a spatial relationship between the sites of glycogen synthesis and the smooth endoplasmic reticulum [19–21]. Although our results do not elucidate the nature and composition of these sites, they do provide information on their intracellular location in distinct metabolic conditions. The de novo synthesis of glycogen in muscle cells involves an initiator enzyme, named glycogenin [22,23], which is expressed in a molar 1:1 ratio with respect to GS and remains covalently bound to the mature glycogen molecule [24]. However, the role of glycogenin in liver is more controversial. In this tissue, the total abundance of this enzyme is much smaller than in muscle, such that the number of glycogen particles in liver far exceeds the number of glycogenin molecules. Thus, most glycogen particles do not have a glycogenin protein attached [25]. Furthermore, 1 h after oral glucose administration, glycogenin activity can be recovered unchanged from rat liver extracts, indicating that this protein is not incorporated into glycogen, even though the concentration of the polysaccharide substantially increases in this period [26]. Therefore, whether the resynthesis of hepatic glycogen after a substantial depletion involves the action of glycogenin or whether small glycogen particles are the primers for the replenishment of the polysac-

charide is still open to discussion [26]. However, our results show that in hepatocytes isolated from fasted rats the particles that can give rise to new glycogen synthesis concentrate near the plasma membrane and, as glycogen deposits grow, move towards the interior of the cell.

We have previously shown in isolated [3] and cultured [4] hepatocytes that GS presents a uniform cytoplasmic distribution in the absence of glucose and when the glycogen content of the cells is low. In both systems, the addition of glucose to the incubation medium leads to concentration of the enzyme in the proximity of the plasma membrane. However, the immunolocalisation of GS in cultured hepatocytes shows that the enzyme remains bound to its substrate and product and presents an intracellular distribution that closely resembles that of glycogen. Upon incubation with glucose, the patches where the enzyme accumulates grow towards the interior of the cell together with glycogen deposits [4]. In contrast, in isolated hepatocytes, immunoreactive GS exhibits a clear accumulation in the form of a narrow ring at the plasma membrane after 30 min incubation with 30 mM glucose [3], when glycogen particles are found scattered throughout the cytoplasm. However, the results presented here indicate that there must be a substantial amount of GS in interior locations of the hepatocyte after initial stages in the synthesis of glycogen and a substantial amount of the polysaccharide is already present. In this situation, it cannot be ruled out that the incorporation of glucose units to glycogen molecules is the result of a futile cycle which involves the simultaneous degradation and re-synthesis of the polysaccharide. Under certain circumstances, GS and glycogen phosphorylase can act simultaneously, leading to an exchange of glucose units between glycogen and the medium [27]. However, this observation does not invalidate our main conclusion that glycogen synthesis becomes gradually active from the periphery of the hepatocyte towards the interior, as the incubation with glucose proceeds.

In conclusion, our results indicate that the synthesis of glycogen follows a spatially ordered pattern in the hepatocyte, but also suggest that the degradation of the polysaccharide is an ordered process. The glycogen phosphorylase-mediated phosphorolysis of glycogen might proceed exclusively from the interior to the exterior of the hepatocyte or simultaneously throughout the cytoplasm. However, in the latter case, remaining glycogen particles must move towards the hepatocyte periphery as the degradation of the polysaccharide progresses, in such a way that after an extensive depletion, the very few remaining glycogenogenic particles are located near the plasma membrane. The observation that the molecules of liver glycogen are synthesised in a defined order and degraded in the reverse order, such that glucose units incorporated first are released last during glycogenolysis and vice versa [28], favours the latter hypothesis.

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