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Random, presumably hydrolytic, and lysosomal glycogenolysis in the livers of rats treated with phlorizin and of newborn rats

Pierre DEVOS and Henri-Géry HERS
Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, UCL 75.39 avenue Hippocrate 75, B-1200 Bruxelles, Belgium

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1. The glycogen formed in the livers of adult rats was labelled by injection of [1-^{14}C]galactose soon after initiation of re-feeding after starvation. The rats were anaesthetized 4h later and glycogenolysis was induced by giving them a mixture of glucagon and insulin. In confirmation of previous work [Devos & Hers (1979) *Eur. J. Biochem.* **99**, 161–167], there was a delay in degradation of the labelled glycogen by comparison with total glycogen. This pattern is considered as characteristic of an ordered glycogenolysis. Treatment of rats with phlorizin abolished the difference between the fate of labelled and total glycogen, causing, therefore, a random glycogenolysis. 2. Foetal liver glycogen was made radioactive by injecting [^{14}C]glucose into the mother at the 19.5 day of gestation, i.e. at the time when this glycogen starts to be synthesized. During the postnatal degradation of this glycogen, radioactive and total glycogen were degraded at approximately the same rate, indicating that glycogenolysis occurred at random. In contrast, when puromycin was injected into the newborn rats, there was a delay in the degradation of the labelled glycogen as compared with that of total glycogen, as currently observed in the normal adult liver. 3. These data are discussed in relation with the fact that glycogen-filled vacuoles are currently seen in the livers of adult rats treated with phlorizin, and also in the neonatal livers, and that puromycin is known to cause the disappearance of these autophagic pictures in the liver of newborn rats. It is suggested that random glycogenolysis occurs through hydrolysis by the lysosomal acid α-glucosidase, in the course of autophagy.

Most animal cells possess two mechanisms for the degradation of glycogen. The first one is phosphorolytic and has been extensively studied; it requires the participation of phosphorylase, amylo-1,6-glucosidase and phosphoglucomutase; it is followed by glycolysis or, in the liver and the kidney, by the formation of glucose through the action of glucose 6-phosphatase. The second mechanism is hydrolytic and lysosomal; it involves only one enzyme, the acid α-glucosidase (Lejeune *et al.*, 1963; Jeffrey *et al.*, 1970). Type II glycogenosis is caused by the deficiency of the latter enzyme (Hers, 1963) and is characterized by the intravacuolar deposition of a large amount of glycogen in most cell types (Baudhuin *et al.*, 1964). The severity of this pathological condition indicates that lysosomal glycogenolysis is normally operative and plays an important role in most tissues. This role appears to be not so much to provide glucose to the cells as to destroy the glycogen which has been engulfed in the vacuolar system during the process of autophagy (Hers & de Barsy, 1973).

There are very few normal or experimental preparations in which glycogen-filled vacuoles, similar to those which are characteristic of type II glycogenosis, have been seen on ultrastructural examination of liver. One of them is the liver of adult rats treated with phlorizin (Becker & Cornwall, 1971). Another one is the liver of the newborn mouse (Jézéquel *et al.*, 1965) or rat (Phillips *et al.*, 1967), which contains a large amount of glycogen at the time of birth and degrades most of it in the following hours. A morphometric analysis of the liver in the postnatal period (Kotoulas & Phillips, 1971) has revealed that the amount of glycogen present in autophagic vacuoles was decreased by more than 40-fold after puromycin was given. Puromycin also decreased the size of lysosomes but

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markedly increased glycogenolysis. Up to now, the problem of lysosomal glycogenolysis has not been investigated by biochemical methods.

We have reported that the phosphorolytic degradation of glycogen in the liver *in vivo* or in a cell-free system follows an order in which the molecules of glycogen which have been synthesized first are degraded last and vice versa (Devos & Hers, 1979). In contrast, this order is expected not to be found when glycogen is degraded by the lysosomal α-glucosidase, since autophagy is believed to occur at random.

The purpose of the present work was to investigate if the postnatal glycogenolysis as well as the glycogenolysis induced by phlorizin occurs at random or is ordered.

Materials and methods

Experiments with adult rats

We have used the general procedure previously described (Devos & Hers, 1979) for early labelling of glycogen upon re-feeding of fasted rats and for induction of glycogenolysis by glucagon and insulin. Phlorizin by itself was unable to decrease the concentration of glycogen in the liver during re-feeding of fasted rats, presumalby because glycogen synthesis is very active in this condition. The procedure was as follows: Wistar rats weighing about 250g were fasted for 40h and re-fed. Ten minutes after the initiation of refeeding, they received an intraperitoneal injection of [1-^{14}C]galactose (0.1mg, 0.02μCi/g body wt.) mixed with unlabelled glucose (0.5mg/g). Four hours later phlorizin (1mg/g body wt. dissolved in a solution containing 0.15M-NaCl and 20% dimethyl sulphoxide) or a corresponding amount (0.5ml/100g body wt.) of the solvent solution was given subcutaneously. The rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (7.5mg/100g body wt.), their livers were exposed and samples (about 0.3g) were cut off and immediately quick-frozen between aluminium blocks precooled in liquid nitrogen (Wollenberger *et al.*, 1960). Glycogenolysis was then induced by subcutaneous injection of glucagon (30μg/100g body wt.) and insulin (1unit/100g body wt.) 30min after phlorizin or its solvent was given. Other liver samples were taken and frozen at time intervals as indicated in Fig. 1. The frozen samples were weighed and dropped into 1ml of 5M-KOH for the isolation of glycogen.

Experiments with newborn rats

Rats of the Wistar strain were used. Pregnant mothers were dated by knowing the time of fertilization (±6h) by the presence of a copulation plug. Carrier-free [^{14}C]glucose was given to the mother at day 19.5 of gestation by two subcutaneous injections (15μCi in each) given at 1h intervals. The foetuses were delivered by caesarean

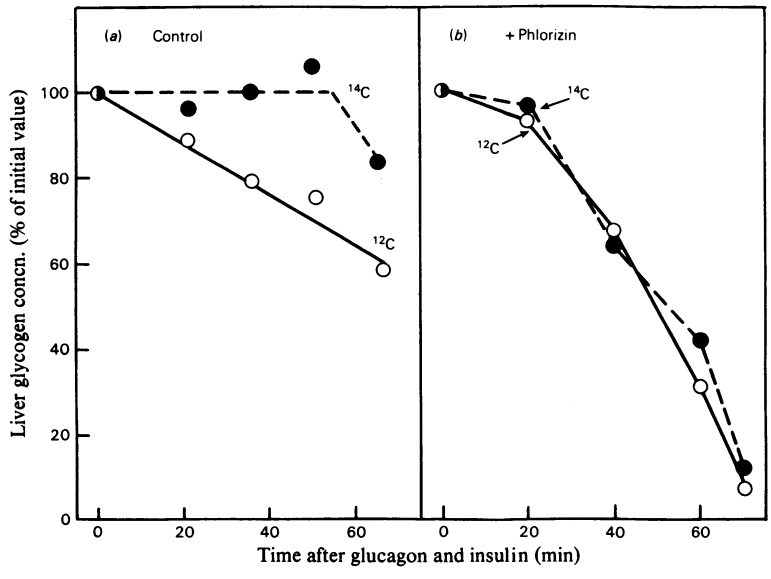

Fig. 1. *Degradation of glycogen in the livers of adult rats treated or not with phlorizin*

Concn. of glycogen at time zero was equal to 3.5% in Fig. 1(*a*) and to 2% in Fig. 1(*b*). Its sp. radioactivity approximated 2000c.p.m./mg in both experiments. Phlorizin (Fig. 1*b*) or its solvent (Fig. 1*a*) control were given 30min before glucagon and insulin. ●---●, intraperitoneal injection of [1-^{14}C]galactose mixed with unlabelled glucose; O——O, unlabelled galactose.

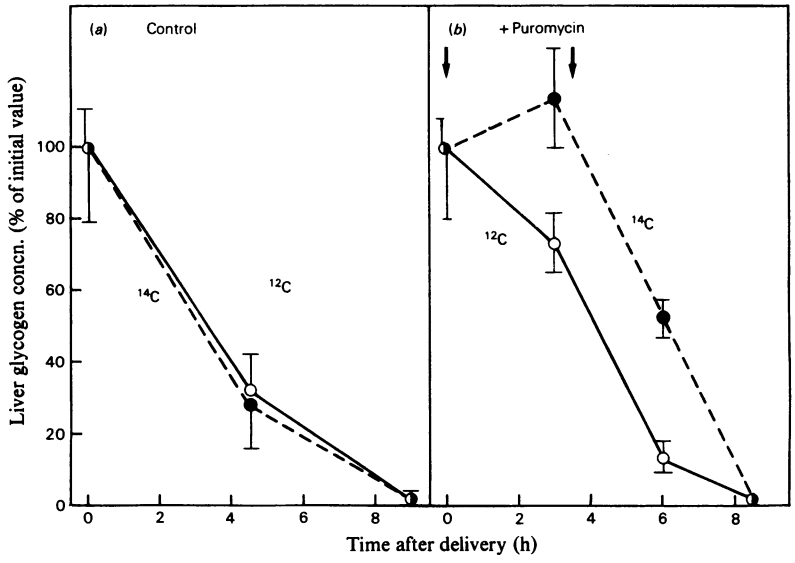

Fig. 2. *Degradation of glycogen in the livers of newborn rats treated or not with puromycin*
Concn. of glycogen at time zero was equal to 8.1% in Fig. 2*a* (control) and 6.2% in Fig. 2*b* (puromycin). Its sp. radioactivity approximated 1000c.p.m./mg in both cases. 0.4mg of puromycin was given intraperitoneally at time zero and at 3h as indicated by the arrows. ●---●, subcutaneous injection of [^{14}C]glucose; O——O, unlabelled glucose. Values shown are means + S.E.M. ($n = 3$).

section at day 21.5 after decapitation of the mother. Puromycin (0.4mg dissolved in 0.05ml of 0.15M-NaCl and adjusted to pH7.0 with NaOH) was injected intraperitoneally. The animals were maintained at 37°C in moistened air and killed at the time indicated in Fig. 2. Their livers were weighed and dropped into 1ml of hot 5M-KOH for the isolation of glycogen.

Other methods and source of chemicals

Acid α-glucosidase was assayed with maltose as substrate as described by Hers (1963). One unit of this enzyme hydrolyses 1μmol of maltose/min in the conditions of the assay. Phlorizin and puromycin were from Sigma Chemical Co., St. Louis, MO, U.S.A., and dimethyl sulphoxide from E. Merck, Darmstadt, Germany. The source of other chemicals and reagents as well as the experimental procedures were as described previously (Devos & Hers, 1979).

Results

Experiments with adult rats given phlorizin

In these experiments, [^{14}C]galactose was injected soon after the initiation of re-feeding of fasted rats. Since the major part of this radioactive precursor has disappeared from the blood within 1h after being given (Devos & Hers, 1979), the glycogen that was synthesized in the liver during the following 3h was unlabelled and the labelling could be considered as specific for the early synthetized glycogen. Fig. 1(*a*) confirms that, when glycogenolysis was induced by giving insulin and glucagon, there was a delayed degradation of the radioactive glycogen. Fig. 1(*b*) shows that, in contrast, total and radioactive glycogen were degraded at nearly the same rate when phlorizin had been given 30min before the initiation of glycogenolysis.

Experiments with newborn rats

In this series of experiments, glycogen was made radioactive by injecting the mother with [^{14}C]-glucose at day 19.5 of the gestation period, i.e. when synthesis of the foetal liver glycogen begins. Since glycogen synthesis continued for 2 days from unlabelled precursors, the labelling was specific for the early synthesized glycogen. Fig. 2(*a*,*b*) show that the postnatal degradation of glycogen followed a very different pattern according to whether or not the newborn rats had been given puromycin. In the control animals (Fig. 2*a*), radioactive and total glycogen were degraded at the same rate. In contrast, the effect of puromycin (Fig. 2*b*) was to cause a delayed degradation of the radioactive glycogen according to a pattern which is characteristic of the normal adult liver (see Devos & Hers, 1979, and also Fig. 1*a*).

It has been checked that that activity of acid α-glucosidase in the livers of newborn rats was the

same as in the adult livers. It was in both cases close to 0.3 unit/g, when maltose was used as a substrate.

Discussion

In a previous publication (Devos & Hers, 1979) we have shown that the phosphorolytic degradation of glycogen in the liver proceeds according to a definite order in which the molecules synthesized last are degraded first and vice versa. This ordered degradation was not observed in the brown adipose tissue, in which glycogen is monoparticulate, and may, therefore, be related to the polyparticulate (α, in the nomenclature of Drochmans, 1962) structure of liver glycogen (Devos & Hers, 1980).

We have now observed two conditions in which the degradation of liver glycogen was not ordered but occurred at random. This random degradation was not caused by a lack of ordered synthesis, since an ordered degradation of the same glycogen could be obtained under other experimental conditions. Indeed, in the livers of adult rats, the random degradation occurred under the action of phlorizin whereas, as already shown in a previous publication (Devos & Hers, 1979), it was ordered when this action was omitted. In the livers of newborn rats, the spontaneous glycogenolysis occurred at random whereas the glycogenolysis observed after puromycin was given was ordered. From these data, one can conclude that there exist in the liver two patterns of glycogenolysis which are independent of the mechanism by which glycogen has been synthesized.

The respective roles of phlorizin and of the hormonal treatment in the induction of the glycogenolysis shown in Fig. 1(*b*) are not clear. One effect of glucagon is obviously to inactivate glycogen synthase and, doing so, to arrest glycogen synthesis which, in re-fed rats, occurs at a rate of approx. 1%/h (Devos & Hers, 1979). Indeed, this very intense synthesis could mask the glycogen breakdown induced by phlorizin. Two other known effects of glucagon are to activate glycogen phosphorylase and also, as described by Deter & de Duve (1967), to induce autophagy. There is, however, a general agreement in the literature that the glucagon-induced glycogenolysis is phosphorolytic rather than autophagic. The comparison between Figs. 1(*a*) and 1(*b*) indicates clearly that the effect of phlorizin has been to change the pattern of glycogen breakdown from ordered, i.e. phosphorolytic, to random.

The two experimental conditions in which we have observed a random glycogenolysis in the liver, i.e. the liver of adult rat given phlorizin and the neonatal liver, are also those in which the ultrastructural analysis had revealed the presence of glycogen-filled autophagic vacuoles. One is, therefore, tempted to assume that this random degradation occurs by autophagy. A further parallelism between the results of the ultrastructural analysis of the liver and our biochemical data, is given by the puromycin experiment. Indeed, puromycin is known to activate phosphorylase (Hofert & Boutwell, 1966) and also to prevent the formation of autophagic vacuoles in the liver of newborn rats (Kotoulas & Phillips, 1971). It is remarkable that, under the same circumstance, glycogenolysis was sequential.

The existence of a hydrolytic and lysosomal pathway for glycogen degradation is known by the existence of type II glycogenosis. Indeed, in this very severe disease, there is a complete deficiency of a lysosomal acid α-glucosidase (Hers, 1963) accompanied by an intralysosomal storage of glycogen (Baudhuin *et al.*, 1964). Until now, there was no biochemical method to distinguish between phosphorolytic and hydrolytic degradation of glycogen *in vivo*; consequently, the relative importance of the two glycogenolytic mechanisms has been difficult to ascertain. The present work is a first attempt in this direction.

Although our observations are suggestive of a lysosomal degradation of glycogen in the adult liver, under phlorizin treatment, and in the neonatal liver, it must be recognized that the amount of acid α-glucosidase currently measured in the liver (about 0.3 unit/g) does not seem to be sufficient to account for the rate of glycogenolysis reported in Fig. 2 (more than 1 μmol of glucose formed/min and per g). One has, therefore, to assume that acid α-glucosidase might be about three times more active *in vivo* than it is *in vitro*. Several factors capable of influencing the activity of the purified acid α-glucosidase have been described (Jeffrey *et al.*, 1970; Palmer, 1971).

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