Disturbance of lysosomal glycogen metabolism by liposomal anti- α -glucosidase and some anti-inflammatory drugs

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The size-distribution of liver glycogen was shown to be distinctly affected by the anti-inflammatory drugs salicylate and indomethacin. By measurement of the incorporation of radioactive glucose into glycogen, salicylate was shown to have a depressing effect on overall liver glycogen metabolism. These effects appear to arise from the stabilizing of the lysosome by the drugs. The incorporation, via liposomes, of purified anti-1,4- α -glucosidase antibodies into the liver lysosomes causes a distinct decrease in 1,4- α -glucosidase activity and in the content of high-molecular-weight glycogen. These changes are increased by prolonged liposomal antibody treatment and suggest that a possible feedback control mechanism operates in the incorporation of glycogen into lysosomes. These experiments may be useful as a model of glycogen turnover and its failure in glycogenosis type II (Pompe's disease).

A series of recent papers (Lüllman-Rauch, 1981*a,b*, 1982) have described how glycogenosis type II (Pompe's disease) may be mimicked in rats by intraperitoneal injection of the 1,4- α -glucosidase inhibitor Acarbose (Bay g 5421; Schmidt *et al.*, 1977; Puls *et al.*, 1980; Padilla *et al.*, 1981). This pseudotetrasaccharide appears to enter the liver lysosomes by fluid-phase endocytosis followed by lysosomal fusion (Silverstein *et al.*, 1977; Daems *et al.*, 1969). The effect of this inhibitor treatment was monitored by histochemistry and cytochemistry, and a mechanism of action was proposed (Lüllman-Rauch, 1982).

It has previously been shown by independent methods that in the liver, and some other tissues, a portion of the cellular content of glycogen is associated with the lysosomes (Geddes & Stratton, 1977*a*; Knecht & Hernandez, 1978; Brown *et al.*, 1978; Rybicka, 1981). Further, this glycogen is distinguished from that in the cytosol, to various extents, by size, structure and incorporation of radioactive precursors of glycogen synthesis (Geddes & Stratton, 1977*a,b*; Geddes *et al.*, 1977*a,b*; Chee *et al.*, 1983). In addition, lysosomes have been reported to have been stabilized by anti-inflammatory drugs, although contradictory results may be obtained because of the difficulties associated with the preparation of stable consistent lysosomal samples *in vitro* (Ignarro, 1971*a,b*). The experiments reported below are an attempt, based on combining the above observations, to test whether glycogen could be used as a 'monitor' of cellular lysosomal activity *in vivo*.

Later experiments, designed to test further the sensitivity of glycogen, were based on the fact that the enzyme involved in the intralysosomal breakdown of glycogen is 1,4- α -glucosidase (EC 3.2.1.3). The genetic lack of this enzyme results in Pompe's disease (glycogenosis type II) (Hers & de Barsy, 1973). Therefore antibody to this enzyme was prepared, encapsulated in liposomes and injected into test animals in an attempt to disturb lysosomal carbohydrate metabolism specifically (Gregoriadis, 1974; Ryman, 1974; Roerdink *et al.*, 1976). Slight differences in the physical properties of glycogen from post-mortem glycogenosis type II liver have previously been reported (Iwamasa *et al.*, 1979), but must be ignored, like other reports based on post-mortem tissue (Edstrom, 1972), because of the rapid non-uniform degradation of glycogen after death (Geddes & Rapson, 1973).

Materials and methods

Glycogen was extracted from the liver of rabbits or rats as described previously (Geddes & Stratton, 1977*a,b*). Fractions were prepared on sucrose density gradients and assayed by using an iodine/iodide reaction (Geddes & Rapson, 1973). The molecular size of the fractions was determined as described previously (Geddes *et al.*, 1977*a*), and all calculations were performed on mini-computers. All results presented are averaged from three separate determinations, the average variation in the concentration scale being typically less than $\pm 2\%$.

Samples of sodium salicylate or indomethacin, in

iso-osmotic saline (0.9% NaCl), were injected intraperitoneally into the rats (male white Wistar, 200–250 g). In radiolabel-incorporation experiments approx. 0.08 mCi of [^{14}C]glucose (230 Ci/mol; The Radiochemical Centre, Amersham, Bucks., U.K.) was mixed with the salicylate solution before injection.

Differences in the glycogen yield (as percentage of the wet weight of liver) were random and not obviously related to any experimental procedure employed, e.g. injection of drugs.

Acid 1,4- α -glucosidase activity was assayed at pH 4.0 with 4-methylumbelliferyl 1- α -D-glucopyranoside (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) as a substrate (den Tandt, 1972; Willcox & Rattray, 1979).

The enzyme was purified from a homogenate of 14 adult rat livers by a dextran absorption method (Auricchio *et al.*, 1968), with an additional purification step involving adsorption on DEAE-cellulose (25 cm $\times$ 2.5 cm column; 5 mM-Tris/HCl buffer, pH 7.0, containing 2 mM-EDTA and 5 mM-2-mercaptoethanol). The enzyme was eluted by a linear salt gradient (0–250 mM-NaCl) and was judged pure by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Approx. 2 mg of the purified enzyme was used to raise rabbit antiserum. The immunogen was dissolved in 0.75 ml of deionized water, thoroughly emulsified with 0.75 ml of Freund's complete adjuvant and divided into three equal portions. These were administered to the rabbit by multiple subcutaneous injection at intervals of 2 weeks. Blood samples (10–15 ml) were then taken from marginal ear veins at subsequent successive 2-week intervals over the following 16 weeks. The antiserum inhibited enzymic activity, and gave a single precipitin line with the purified enzyme in double-diffusion analysis. An immune γ -globulin fraction was prepared from the antiserum by $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-cellulose chromatography (Deutsch & Fahey, 1967). Approx. 3 mg of this fraction was used to make (Gregoriadis *et al.*, 1971) a 1 ml liposome suspension (de Barsey *et al.*, 1975) (phosphatidylcholine, cholesterol and stearylamine in the molar proportions 7:2:1; approx. 10 mg of lipid/ml of liposome suspension). Liposomes prepared by this method are small multilamellar (Gregoriadis *et al.*, 1971) and lie in size between the small unilamellar and large multilamellar liposomes produced under different conditions. Liposomes were fractionated by Sepharose 4B gel filtration, to remove any contaminating multilamellar liposomes etc., and found to contain about 5% of the globulin (measured by disruption of the liposomes by 0.1% Triton X-100 and comparison with starting material). The liposome suspension was then injected equally into the tail veins of four adult rats. The residual γ -globulin was

dialysed, freeze-dried and used to make a fresh liposome suspension weekly, which was then injected into the remaining animals. Animals were killed over successive weeks, and either their whole liver glycogen was extracted or the glycogen from the lysosome-enriched fraction (Geddes & Stratton, 1977a).

Results

Fig. 1. shows the effect on the molecular-weight distribution of liver glycogen of treatment with the anti-inflammatory drugs salicylate and indomethacin. These drugs, which appear to stabilize the lysosomal membrane (Ignarro, 1971a,b), decrease the amount of high-molecular-weight as compared with low-molecular-weight glycogen. This induced metabolic inhomogeneity in glycogen turnover mimics the effect, for example, of a starvation/re-feeding cycle in normal rabbits (Geddes, 1971; Geddes & Stratton, 1977b). It seems possible that the observed effect arises from drug stabilization of the lysosomal membrane and the consequent decreased ingestion, by whatever mechanism, of high-molecular-weight glycogen into the lysosome. This is consistent with the observation (Geddes & Stratton, 1977a) that it is this glycogen that is normally associated with liver lysosomes.

Further experiments with increased doses of salicylate had [^{14}C]glucose administered simultaneously, since this has proved to be a sensitive measure of glycogen metabolism (Geddes & Stratton, 1977b). The effect of the salicylate on the molecular-weight distribution of the glycogen paralleled that shown in Fig. 1, but in addition caused severe disturbance to the incorporation of radioactive glucose into glycogen, as depicted in Fig. 2. There was increased incorporation of radioactivity into all sizes of glycogen, but the effect was most marked again in the high-molecular-weight fractions (fraction 8 has M_r approx. 400×10^6 – 500×10^6). Salicylate clearly has an overall depressing effect on glycogen metabolism, but the effect is most marked on high-molecular-weight material. Increasing dosage increases the overall effect evenly. It seems likely therefore that the decreased removal of glycogen into the lysosome has a depressing effect on continued glycogen synthesis, presumably through the well-known cascade control system (Cohen, 1982).

Experiments on the incorporation of anti-1,4- α -glucosidase antibody into liver cells by means of a liposomal carrier were also attempted. It is generally accepted now that the liver cells take up relatively most of liposomal material injected into the bloodstream, although its distribution between the parenchymal and the Kupffer cells remains unclear (Scherphof *et al.*, 1980). We have con-

Fig. 1. Molecular-weight distributions of liver glycogens from rats that had been intraperitoneally injected with 20 mg of salicylate (○) or 0.2 mg of indomethacin (●) as compared with an untreated animal (■)
For experimental details see the text. All results were averaged from three determinations and are normalized to aid comparison. (Average errors in the relative concentration scale are less than ±2%.)

Fig. 2. Effect on the incorporation of [¹⁴C]glucose into glycogen when salicylate (60 or 120 mg) is injected simultaneously
For experimental details see the text. Results are expressed as the ratio of the radioactivity (c.p.m.) incorporated per mg of glycogen in each fraction from the untreated animal (N) as compared with the treated animals (60 and 120) (○, N/60 incorporation ratio; ●, N/120 incorporation ratio). Additionally, a comparison between the two treated animals is shown (■, 60/120 incorporation ratio). Fractions were collected as described in Geddes & Stratton (1977a).

firmed (Table 1) that 1,4- α -glucosidase activity is decreased on introduction of the liposome-entrapped specific antibody, and have also shown that the absolute amount of high-molecular-weight glycogen associated with the lysosome-enriched fraction (Geddes & Stratton, 1977a) increases correspondingly (Table 1). However, in whole glycogen, as shown in Fig. 3, there is a greater increase in the low-molecular-weight region, resulting in a decrease in the relative amount of high-molecular-weight glycogen as compared with its lower-molecular-weight counterpart. The size of this relative increase is clearly indicated in Table 2. These results show similarities to the effects induced by the administration of anti-inflammatory drugs, as shown in Fig. 1. Again the results suggest that there is some form of feedback control on glycogen uptake when the normal function of the lysosome is disturbed.
Although we have presented the results of a single series of liposomal antibody experiments, we have obtained similar results in another, completely independent, series with a different anti-1,4- α -glucosidase preparation.

Table 1. Effect of liposomal antibody on the lysosomal glycogen concentration and 1,4- α -glucosidase activity			
Lysosomal glycogen was prepared as described by Geddes & Stratton (1977a). For other experimental details see the text. Control glycogens were isolated from untreated livers, but otherwise were subjected to the same procedures as the experimental tissues. The decrease in 1,4- α -glucosidase activity was assayed as the relative decrease in the specific activity of the 1,4- α -glucosidase in experimental animals relative to control (untreated) animals, divided by the same ratio for β -glucuronidase in the same animals.			
Duration of treatment ...	1 week	2 weeks	3 weeks
Increase in lysosomal glycogen as compared with control preparation (%)	4.2	5.2	25.0
Decrease in 1,4- α -glucosidase activity (%)	10.3	19.6	22.9

Table 2. Variation in various molecular size ranges of glycogen after treatment with liposomal-enclosed antibody for a number of weeks
For experimental details see the text. Results are expressed as the percentage variation, with respect to normal (untreated) glycogen distribution, of material within each size range.

10 ⁻⁶ × M _r range	Duration of treatment ...	Variation (%)		
		1 week	2 weeks	4 weeks
70–90		+2.64	+5.99	+11.99
190–380		–1.03	+3.41	+18.27
380–840		–8.74	–5.58	–8.46
>840		+0.80	–5.61	–9.33

Fig. 3. Effect on the molecular-weight distribution of rat liver glycogen of treatment with liposomes containing anti-1,4- α -glucosidase antibody

For experimental details see the text. Duration of treatment with liposomes containing the antiserum: ----, none (control); Δ , 1 week; $\bullet$, 2 weeks; $\circ$, 4 weeks. Results are averaged from three determinations and are normalized to aid comparison.

Discussion

Our results with antibodies to 1,4- α -glucosidase are complementary to those obtained by Lüllman-Rauch (1981a,b, 1982) with a synthetic 1,4- α -glucosidase inhibitor. However, we believe that we have extended the significance of this experimental approach insofar as: (a) the latter administered an 1,4- α -glucosidase inhibitor of which only a minor part would have ended up in the liver cell and its lysosomal apparatus (after intravenous dosage in humans about 90% of the inhibitor is excreted in the urine within 24 h; Puetter, 1980), whereas our specific antibody, encapsulated in liposomes, was targeted for the liver lysosomal system (Gregoriadis, 1974; Ryman, 1974; Roerdink *et al.*, 1976). (Table 1), and possibilities of serious artifactual metabolic disturbance can be ignored (injection of 'empty' liposomes had no significant effect on the amount of glycogen, its molecular-weight distribution or on enzymic activities); (b) our studies are concerned with the variation in the effects within the vast molecular size range of liver glycogen molecules, i.e. alterations to the well-known metabolic inhomogeneity of liver glycogen (Stetten *et al.*, 1956; Geddes, 1971; Geddes & Stratton, 1977a,b) and not

just with the gross overall glycogen content as viewed histochemically and cytochemically.

Further, these results provide at least a partial explanation for the apparent molecular ordering of the synthesis and degradation of glycogen in the liver (Devos & Hers, 1979, 1980) in that the molecules synthesized last are degraded first, and vice versa.

Our results show categorically that disturbance of normal liver lysosomal function, whether by interference with the membrane (Ignarro, 1971a,b) (Figs. 1 and 2) or by specific enzymic inhibition (Fig. 3 and Tables 1 and 2) of a single lysosomal enzyme, causes acute disturbance to the overall cellular carbohydrate metabolism, not just to the lysosomal portion. It is clear that, despite the absolute increase in glycogen associated with the lysosome (Table 1), the relative amount of this high-molecular-weight material actually decreases (Table 2), indicating that synthesis is continuing but that there is a previously undescribed 'feedback' on the process by which high-molecular-weight glycogen is encapsulated in the lysosomes. Since the high-molecular-weight glycogen is built on a protein backbone (Krisman & Barengo, 1975; Geddes & Stratton, 1977b; Chee & Geddes, 1977; Geddes *et al.*, 1977b), and in fact may be distinguished from its low-molecular-weight counterpart by its high protein content (Chee & Geddes, 1977), it would seem likely that the synthesis and packaging of the large glycogen particles into the lysosomes proceeds by a process similar to that used in the synthesis of glycoprotein (Palade, 1975; Lodish *et al.*, 1980). Our results show that disturbance of normal processing at a later stage of the pathway (illustrated by Lüllman-Rauch, 1982, Fig. 10a) results in the accumulation particularly of low-molecular-weight glycogen, which the normal phosphorolytic pathway, without external hormonal stimulus (Cohen, 1982), fails to clear. It is also possible that the slow clearance of material that disturbs lysosomal metabolism, such as Acarbose (Lüllman-Rauch, 1981a, 1982), may exacerbate this effect.

The fact that disturbance of the non-phosphorolytic pathway of glycogen degradation clearly affects the phosphorolytic degradation, even with the relatively small amounts of antibody used in these experiments, may help to explain the observation that in type II glycogenosis, where lysosomal glycogen storage is massive because of the genetic lack of 1,4- α -glucosidase (Hers & de Barsey, 1973), normal cell function is impaired to such an extent that most patients die in early childhood (Huijing, 1973).

The two experimental approaches used in these studies involve disturbance of lysosomal integrity and metabolic function respectively, and affect the glycogen fraction that has been previously shown to be characteristic of lysosomes (Geddes & Stratton,

1977a). Thus it may be feasible to use the measurement of total glycogen distribution on a molecular-weight basis as a convenient monitor of lysosomal behaviour *in vivo*.

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