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MOLECULAR AND METABOLIC HETEROGENEITY OF LIVER GLYCOGEN*

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ABSTRACT

On refeeding after starvation, the resynthesis of rabbit-liver glycogen proceeds inhomogeneously and over-produces material of low molecular weight. The fate of radioactivity incorporated into glycogen from D-glucose-^{14}C can be explained if glycogen of high molecular weight is synthesised on a protein backbone. Confirmation of this view is given by the effect upon glycogen of reagents that break disulphide bonds; these cause loss of the polysaccharide of high molecular weight. Buoyant densities of glycogens are found to be independent of molecular weight and even of extensive degradation. It is concluded that glycogen synthesis proceeds by two routes; one results in the production of polysaccharide of high molecular weight which has a protein backbone capable of forming disulphide bonds, and another results in the production of polysaccharide of low molecular weight which has either no protein backbone or a protein backbone that is incapable of forming disulphide bridges. Apart from size, the two species are physicochemically indistinguishable.

INTRODUCTION

It is well-established that the metabolism of liver glycogen is a heterogeneous process, in that not all of the vast range of sizes of glycogen molecules are synthesised and degraded at the same rate[1,2]. However, a recent study[3] of the physicochemical behaviour of glycogen sub-fractions has shown that, with the exception of those of very smallest molecular weight, there is no significant variation in properties, such as the frictional ratio or the β function of Scheraga and Mandelkern[4], with molecular size. The differing metabolic rates are therefore not obviously related directly to the conformation of the molecule, although the overall size of the molecule does affect its rate of degradation by alpha-amylase[5], and there are alpha-amylase-resistant regions in glycogen molecules[6]. Post-mortem degradation of glycogen is also a heterogeneous process[7]. Glycogen synthesis takes place on a protein backbone[8], and the significance

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of this process has been clearly discussed by Whelan[9]. We have also recently described[10] the association of liver glycogen of high molecular weight with the lysosome, whereas its counterpart of low molecular weight is found in the cytosol.

The object of the research reported here was to investigate more closely the heterogeneity of liver glycogen and to attempt to correlate some of the observations mentioned above. Further, we noted that if the very large glycogen molecules were built on a protein backbone[8], this would have to be an unusually large protein; glycogen is found[3] as molecules of mass up to $\sim 10^9$ daltons. The spherical β-particles[11] of which these large molecules are composed have molecular weights[3] of $\sim 10^7$. If glycogen is linked to protein, it is likely, from studies of linkages found in glycoproteins and mucopolysaccharides[12], to be through the side chains of serine, threonine, hydroxylysine, or asparagine. A simple calculation shows that, taking account of the content of any of these amino acids in typical proteins (usually $<10\%$ of amino acid residues), the mass of the protein involved would have to be 2×10^5 daltons or even greater. It was therefore decided to examine the way in which the molecular weight distribution of glycogen is affected by reagents that break disulphide bonds.

EXPERIMENTAL

Animals. — Young, adult, male rabbits (New Zealand White) were used in all experiments, and livers were quickly removed after inducing rapid anaesthesia with an overdose of Nembutal (Abbott Laboratories Ltd., Naenae, New Zealand) injected into an ear vein. The tissue was immediately stored in liquid nitrogen before extraction of glycogen.

In experiments involving the incorporation of D-glucose-^{14}C (Radiochemical Centre, Amersham, U.K.), the isotope was diluted in ~ 1 ml of isotonic saline and injected into an ear vein.

Glycogen. — Glycogen was normally extracted by a cold-water method utilising phasic separation in 45% aqueous phenol[7,10,13]. Additionally, for the buoyant-density experiments, extractions were performed with 60% potassium hydroxide[14] at 100°, 10% trichloroacetic acid[1] at 2°, or methyl sulphoxide at room temperature[15].

Glycogen was fractionated on sucrose–density gradients[7,10], and the various sedimentation coefficients were determined by the method of Martin and Ames[16], using a partial specific volume[3] of 0.63 ml/g.

Concentrations were measured by an iodine–iodide method[7] which was insignificantly affected by the presence of up to 50% of sucrose[10].

Diffusion coefficients were determined by laser intensity fluctuation spectroscopy[17,18], and molecular weights were determined by application of the Svedberg equation[3].

Buoyant densities were measured in caesium chloride solutions by a standard method[19].

Disruption of disulphide bonds. — Some samples of purified glycogens were treated with reagents that break disulphide bonds. Solutions of glycogen (~10 mg/ml) in 8M urea (pH 8.5, 0.1M Tris buffer) were treated with excess (0.1 ml/ml) of 2-mercaptoethanol for 30 min. These solutions were then dialysed against running water overnight, or treated with excess (0.3 g/ml) of iodoacetamide and then dialysed.

RESULTS AND DISCUSSION

Effect of post-starvation refeeding on glycogen distribution. — The distributions after sucrose density-gradient centrifugation of glycogens from animals which had been allowed to refeed *ad libidum* for various periods after starvation for 4 days are shown in Fig. 1. The total liver-glycogen contents of these animals are recorded in Table I. It can be seen that the glycogen concentration increases in 12 h to very much more than is normally found in the liver. This unexpected effect was confirmed by separate experiments in additional rabbits. A very similar effect has been reported in studies with rats[20]. It is clear from Fig. 1 that the over-synthesis of glycogen is mainly in the range of low molecular weight, which is another example of metabolic hetero-

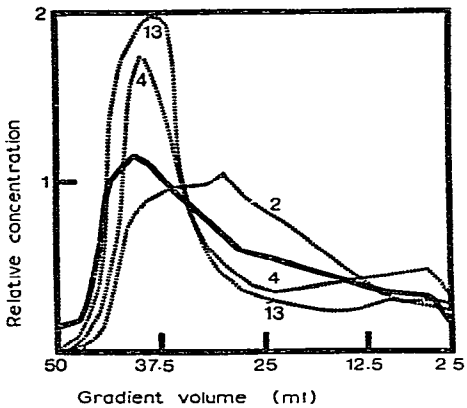

Fig. 1. The effect of refeeding upon the size distribution of glycogens for rabbits that had been starved for 4 days (——, normal animal). The numbers indicate the number of hours of refeeding. (Note: as the gradients were recovered from the bottom of the tubes after centrifugation, the size of the glycogen molecules is inversely related to the gradient volume.)

TABLE I

EFFECT OF REFEEDING ON LIVER-GLYCOGEN CONCENTRATIONS OF STARVED ANIMALS

	4-Day starved animals				*Normal (unstarved)*
Period of refeeding (h)	2	3	4	12	
Glycogen content (% of wet-liver weight)	1.47	4.21	5.31	12.80	5.45

geneity. This result contrasts with the very rapid loss of glycogen of high molecular weight in the post-mortem rat[7]. After starvation, liver glycogen is depleted to ~10% of its normal level, but remains at this level for extended periods of fasting[21]; this material is of low molecular size[2]. The reason for the survival of this glycogen may be related to its dense structure, which impedes access of the degradative enzymes[5,6]. This molecule must be the core upon which the renewed synthesis of glycogen proceeds.

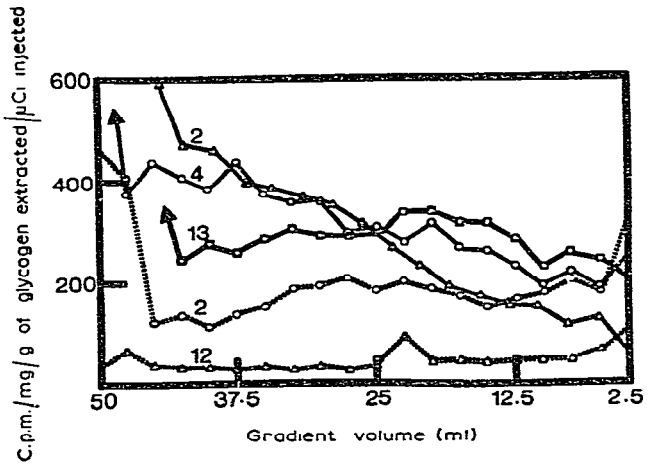

Fig. 2. The redistribution of radioactivity in glycogen molecules. The glycogen was incorporated from D-glucose-^{14}C injected into an ear-vein. Refeeding of starved animals proceeded from the time of injection: ▩ ▩ ▩ ▩, normal animals; ——,starved/refed animals. Numbers indicate the time (h) after injection of radioactivity.

Radioactive incorporation studies. — The results of the injection of ^{14}C-labelled D-glucose into the circulation of rabbits at the end of their starvation period are shown in Fig. 2, and compare favourably with some previously published material[2]. However, the ordinate is expressed as counts/min per milligram of glycogen in the fractions per gram of total glycogen recovered per μCi of radioactivity injected. This expression enables a direct comparison of the radioactivity incorporated to be made between fractions in the same and different samples. The incorporation of isotope into glycogen in normal animals is nearly uniform over most of the fractions, but there is slightly greater incorporation into material of high molecular weight, and a large amount into the two lowest fractions (representing only ~4% of the total glycogen). Similarly, the turnover of the glycogen, as shown by the relative loss of isotope in the 12-h sample, is largely uniform, with the exception of the two smallest fractions whose loss of radioactivity is very large (see also Fig. 3). In contrast, the incorporation of radioactivity into glycogen after starvation is far from uniform. After 2 h of refeeding, the bulk of radioactivity is found in the lower range of molecular weight. After 4 h, the amount of radioactivity in the range of higher molecular weight actually increases;

this trend is continued in the 13-h sample. By 13 h, the distribution of radioactivity parallels that found in normal (*i.e.*, unstarved) animals, but at a significantly higher level. Most of the radioactivity has been preserved in the glycogen during this period, reflecting the overall lack of usage of glycogen as an energy source in this period.

Fig. 3 shows the difference in metabolism between the normal animal and one recovering from a period of starvation. In the normal animal over a 10-h period, there is a fairly uniform loss of radioactivity over the complete range of molecular weights, although there may be a tendency for the material of high molecular weight to be metabolised at a lower rate (see Table II). The metabolic turnover in the liver of the rabbit recovering from starvation is grossly influenced by molecular size as shown in

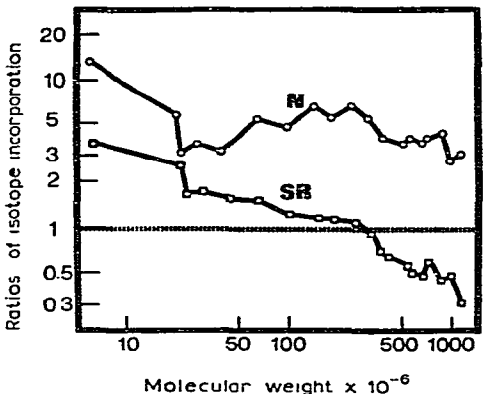

Fig. 3. The turnover of incorporated radioactivity with respect to molecular weight of glycogen. The ratios are calculated from the data in Fig. 2, using the 2-h and 12-h incorporations for normal (N) animals, and the 2-h and 13-h incorporations for starved–refed (SR) animals. Molecular weights of the various fractions were calculated from the Svedberg equation, using diffusion coefficients measured independently by laser dynamic light scattering[3].

TABLE II

FATE OF RADIOACTIVITY INCORPORATION INTO GLYCOGEN IN THE NORMAL AND STARVED/REFED ANIMALS

Molecular weight ($\times 10^{-6}$)	*Ratios of radioactivity*[a]					
	Normal (2 h/12 h)			*Starved/refed (2 h/13 h)*		
	Mean	*S.d.*[b]	*Points omitted*[c]	*Mean*	*S.d.*[b]	*Points omitted*[c]
400–>1000	3.91	0.70	0	0.49	0.10	0
0–400	5.28	1.25	2	1.23	0.31	2
0–>1000	4.67	1.23	2	0.90	0.45	2

[a]Ratios calculated from the data in Fig. 2. [b]Standard deviation. [c]Points were omitted from the calculation when they varied by more than 2 standard deviations from the mean. The total number of points considered was 20.

Fig. 3 and in Table II. Glycogen molecules of mass greater than $\sim 400 \times 10^6$ daltons actually increase their relative radioactivity at the expense of their counterparts of lower molecular weight. The "changeover point", where loss of radioactivity changes to gain, must be related to the association of glycogen of high molecular weight with lysosomes (or lysosome-like organelles)[10].

The preservation of the incorporated radioactivity and its redistribution indicate that the large glycogen molecules are gradually built up from pre-existing, small glycogen molecules, and are not synthesised *de novo* once the cell has built up its stores of small glycogen molecules. If we accept Krisman and Barengo's model[8] in which glycogen synthesis proceeds on a protein backbone, this means that all glycogen molecules synthesised in the first few hours after starvation are formed on the same protein backbone and continue to enlarge on it. The later synthesis which "over-produces" glycogen of low molecular weight (Fig. 1) either proceeds by (*a*) the same mechanism, but cannot continue to enlarge the molecules because of some control mechanism activated by the existing stocks of glycogen of large molecular size; or (*b*) by another mechanism, perhaps by being built upon another, smaller, protein backbone, or else by not having the protein component. The latter seems to be the most likely explanation.

Disulphide bonds and glycogen distributions. — It seemed unlikely (see Introduction) that any protein backbone involved in holding together the large amounts of β-particles found in glycogen of high molecular weight could be a single molecule. Further, it is well known that hydrogen-bond-breaking reagents have no effect on the size-distribution of glycogen molecules[15], indicating that covalent bonds must be involved in holding these massive aggregations together. Therefore, it was decided to test the effect of disulphide bond-breaking reagents upon the distributions, as cystine is well known as a stabiliser of large protein conformations, both at the tertiary and quaternary level.

The effect of treating glycogen with reagents that break disulphide bonds is shown in Fig. 4. Control experiments have been omitted for the sake of clarity; both 8M urea and 8M urea with iodoacetamide caused no significant change in the molecular weight distribution of the glycogen solutions. The sample treated only with 2-mercaptoethanol, which was subsequently removed by dialysis, differs from the original in that there has been some redistribution within the species of high molecular weight. Clearly, only glycogen of high molecular weight is associated with protein that has susceptible disulphide bridges and, as is obvious from the additional effect of iodoacetamide, these may re-form, presumably randomly, when the 2-mercapto-ethanol is removed. The effect of the addition of iodoacetamide to the reduced species, thereby preventing re-formation of disulphide bridges, is to eliminate glycogen of high molecular weight. These results clearly show that glycogen of high molecular weight is built on a protein backbone, and that such protein backbones can be covalently linked to each other by disulphide bonds. These results are in agreement with the final explanation (*b*) offered for our results on the incorporation of radio-active isotopes.

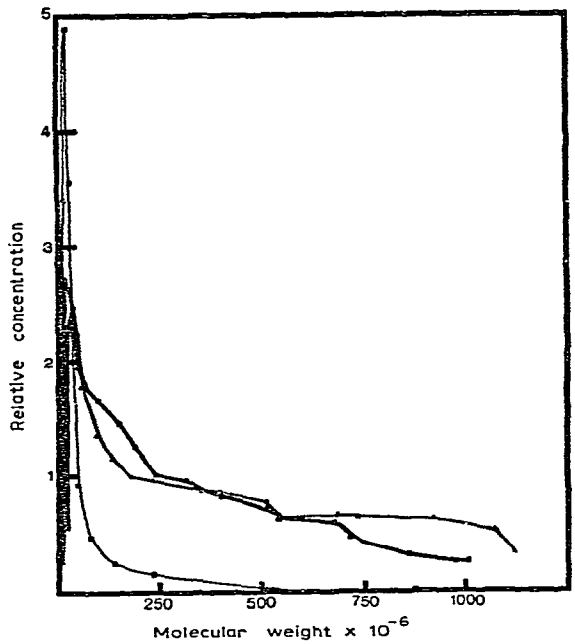

Fig. 4. The effect of disulphide bond-breaking reagents upon the molecular weight distribution of glycogen. Molecular weights were calculated from the Svedberg equation, using the sedimentation coefficients measured in the sucrose-density gradients[16], and diffusion coefficients measured by laser dynamic light scattering[3,17]; ■ ■ ■ ▲ ■ ■ ■, original; ——■——, treated with 2-mercaptoethanol; ——□——, treated with 2-mercaptoethanol and iodoacetamide.

TABLE III

EFFECT OF DISRUPTION OF DISULPHIDE BONDS ON DIFFUSION COEFFICIENTS OF VARIOUS GLYCOGEN SUB-FRACTIONS

$S_{20,w}$ (*Svedberg*)	$D_{20,w}$[a] *for unmodified glycogen* ($cm^2.sec^{-1} \times 10^8$)	$D_{20,w}$[b] *for treated glycogen* ($cm^2.sec^{-1} \times 10^8$)	*Relative conc. of treated glycogen*
16	2.6	3.20	0.06
55	2.47	3.33	0.09
110	3.23	6.58	0.47
170	4.65	7.72	0.72
240	5.80	7.89	1.00
390	5.80	6.56	0.67
580	4.70	4.51	0.31
770	3.82	3.71	0.25
1090	3.12	3.34	0.17
1540	2.73	1.96	0.09

[a]Values interpolated from the data in Ref. 3. [b]Glycogen solution treated with 2-mercaptoethanol and iodoacetamide, as detailed in the experimental Section.

A close analysis of various sub-fractions of 2-mercaptoethanol–iodoacetamide-treated glycogen (Table III), isolated by sucrose-density centrifugation, indicates that most of the material at any particular sedimentation coefficient exhibits diffusion coefficients that are appreciably higher than that of normal glycogen. This property suggests that they have a more compact physicochemical nature than the "normally" synthesised small molecules of glycogen. The significance of this is being investigated[23]

Buoyant-density measurements. — In order to check if the various sizes of glycogen might be distinguishable by a simple physicochemical treatment, the densities of various glycogens and sub-fractions were measured in caesium chloride solutions, and the results are listed in Table IV. It is clear that, even if the glycogen is extracted from the tissue under conditions (hot concentrated alkali, trichloroacetic acid, or methyl sulphoxide)[15,22] that are known to degrade glycogen extensively and some of which (hot concentrated alkali, trichloroacetic acid) would cause practically total destruction of protein, there is no detectable physicochemical variation. The lack of variation in the sub-fractions is in agreement with the results of a recent study of other physicochemical parameters of glycogen sub-fractions[3]. It may be concluded (*a*) that the presence of a small amount of protein does not affect the overall construction of the glycogen molecule, and (*b*) that all sizes of glycogen are constructed in an essentially similar manner.

TABLE IV

BUOYANT DENSITIES OF GLYCOGENS IN CAESIUM CHLORIDE SOLUTION

	Normal	*ICA-glycogen*[a]		*Me_2SO-glycogen*[b]		*KOH-glycogen*[c]
Buoyant density	1.66	1.66		1.65		1.66
	Random sub-fractions of normal glycogen					
	1	*2*	*3*	*4*	*5*	*6*
Buoyant density	1.66	1.65	1.65	1.65	1.65	1.65

[a]Glycogen extracted with cold trichloroacetic acid. [b]Glycogen extracted with methyl sulphoxide [c]Glycogen extracted with hot 60% KOH.

CONCLUSIONS

The foregoing data show that although all the different molecular sizes of glycogen behave in essentially the same physical manner in solution, in agreement with other work[3], they are nevertheless distinguishable on simple chemical grounds, namely, by the presence of disulphide bonds in glycogen of high molecular weight, and not in its counterpart of low molecular weight. Further, this chemical heterogeneity must be related to the metabolic heterogeneity shown by the unusual incorporation and turnover patterns of radioactivity in glycogen isolated from animals recovering from starvation.

From these results, it is tentatively suggested that the two species of glycogen may be synthesised in different regions of the cell, possibly close to the rough and the smooth endoplasmic reticulm. The species synthesised close to the rough endoplasmic reticulum is capable of being enlarged to form material of very high molecular weight, and is formed on a protein backbone. This protein, which will be synthesised by the ribosomes, is capable of forming disulphide bridges that hold the large glycogen molecules together. By contrast, in the region of the smooth endoplasmic reticulum, only glycogen of low molecular weight may be synthesised, because of the lack of protein backbone.

Although the intact glycogens of low and high molecular weight are indistinguishable by buoyant density and other physicochemical parameters[3], the 2-mercaptoethanol–iodoacetamide-treated species have higher diffusion coefficients and are therefore more compact molecules. The reasons for this are not clear, and investigations are continuing[23].

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